



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Avi J. ASHKENAZI, *et al.*

Application Serial No. 09/989,725

Filed: November 20, 2001

For: NUCLEIC ACID ENCODING
PRO1375 POLYPEPTIDES

) Examiner: Hamud, Fozia

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) Art Unit: 1647

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) Confirmation No: 2364

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) Attorney's Docket No. 39780-2730 P1C71

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) Customer No. 35489

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EXPRESS MAIL LABEL NO.: EV 765 982 324 US

DATE MAILED: JANUARY 13, 2006

ON APPEAL TO THE BOARD OF PATENT APPEALS AND
INTERFERENCES APPELLANTS' BRIEF

MS: APPEAL BRIEF - PATENTS

Commissioner for Patents -

P.O. Box 1450

Alexandria, Virginia 22313-1450

Dear Sir:

This Appeal brief is filed in response to the Communication mailed on December 29, 2005 requiring the Appeal Brief to be compliant with the format requirements of 37 C.F.R. §41.37(c). An Appeal Brief was timely filed with the required fees and a 3 month Request for Extension of Time on October 3, 2005 in the above captioned patent application. A Final Office Action was mailed on November 15, 2004 and a Notice of Appeal was filed on May 5, 2005. Appellants hereby request consideration and appeal to the Board of Patent Appeals and Interferences from the final rejection in this case.

The following constitutes Appellants' Brief on Appeal.

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1. REAL PARTY IN INTEREST

The real party in interest is Genentech, Inc., South San Francisco, California, by an assignment of the patent application U.S. Serial No. 09/941,992 recorded November 16, 2001, at Reel 012176 and Frame 0450.

2. RELATED APPEALS AND INTERFERENCES

The claims pending in the current application are directed to a polypeptide referred to herein as "PRO1375". There exists one related patent application, U.S. Serial No. 09/997,573, filed November 15, 2001 (containing claims directed to PRO1375 polypeptides). This application is also under final rejection from the same Examiner and based upon the same outstanding rejections, an appeal of these final rejections is being pursued independently and concurrently herewith.

3. STATUS OF CLAIMS

Claims 119-127, 129-132 and 134-138 are in this application.

Claims 1-118, 128 and 133 are canceled.

Claims 119-127, 129-132 and 134-138 stand rejected and Appellants appeal the rejection of these claims.

A copy of the rejected claims involved in the present Appeal is provided as Appendix A.

4. STATUS OF AMENDMENTS

The claims involved in the appeal have been amended by an amendment filed concurrently with this appeal brief wherein Claims 120 - 124 have been amended to label the subparts of the claims from (a) through (f) correctly and the functional recitations of Claims 119-123 have been amended for clarity. The claims listed in the Appendix incorporate this amendment.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The invention claimed in the present application is related to a nucleic acid that encodes a polypeptide of SEQ ID NO:418 referred to in the present application as "PRO1375"; a nucleic

acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide; a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO:418; the nucleic acid sequence of SEQ ID NO:417, the full-length coding sequence of the nucleic acid sequence of SEQ ID NO:417; or the full-length coding sequence of the cDNA deposited under ATCC accession number 203115 (Claims 124-127 and 129). The PRO1375 polypeptide was shown in the present application to induces proliferation of stimulated T-lymphocytes in a mixed lymphocyte reaction as compared to controls which is set forth in the specification in Example 151. The invention is further directed to polypeptides having at least 80%, 85%, 90%, 95%, or 99% nucleic acid sequence identity to: a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418; a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide; a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO:418; the nucleic acid sequence of SEQ ID NO:417, the full-length coding sequence of the nucleic acid sequence of SEQ ID NO:417; or the full-length coding sequence of the cDNA deposited under ATCC accession number 203115, wherein the polypeptide encoded by said nucleic acid induces proliferation of stimulated T-lymphocytes in a mixed lymphocyte reaction (Claims 119-123).

The cDNA nucleic acid and isolation of clones encoding PRO1375 is described in the specification at, for example, Example 135, page 511 of the specification, in Figure 299 and SEQ ID NO: 417. Hybridization conditions for nucleic acids hybridizing to SEQ ID NO: 417 are discussed on page 275, line 5, page 312, line 23 to page 313, line 4 and Example 139 (Claims 132, 134). The full-length PRO1375 polypeptide is described in the specification at, for example, page 40, line 9-13, page 275, line 1 to page 277, line 3; in Figure 300 and in SEQ ID NO:418. Page 302, lines 28-31 of the specification provides the description for Figures 300 and 299. That PRO1375 is a polypeptide having homology to PUT2, putative protein 2 is described at page 40, line 9-13.

PRO nucleic acid variants having at least about 80% sequence identity to a DNA molecule that encodes the same mature polypeptide encoded by any of the disclosed human cDNAs deposited with the ATCC are described in the specification at, for example, page 284, line 30, to page 285, line 5 and page 308, line 7 to p 311, line 10. Methods for selecting a vector are generally set forth in the specification at, for example, in Examples 140-143 and page 376,

line 12 onwards (Claims 137-138), and describes the expression of PRO nucleic acids in various host cells, including *E. coli*, yeast and Baculovirus-infected insect cells. Methods for selecting a vector are generally set forth in the specification at, for example, page 378, line 8, to page 380, line 11 (Claims 135 and 136).

Example 151 shows that PRO1375 tested positive in the mixed lymphocyte reaction (MLR) assay, demonstrating that PRO1375 is active as a stimulator of the proliferation of stimulated T-lymphocytes, and therefore would have utility in the treatment of conditions where the enhancement of an immune response would be beneficial. In addition, Example 165 shows the ability of PRO1375 to stimulate an immune response and induce inflammation at the site of injection in the skin vascular permeability assay, using the hairless guinea pig injected with the Evans blue dye as a model system.

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

- I. Whether Claims 119-127, 129-132 and 134-138 should be accorded the priority of U.S. provisional application Serial No. 60/144758, filed July 20, 1999.
- II. Whether the data generated in the MLR assay (Example 151) satisfies the utility requirement set forth in 35 U.S.C. § 101 and the "how to use" prong of the enablement requirement set forth in 35 U.S.C. § 112, first paragraph for the invention claimed in Claims 119-127, 129-132 and 134-138.
- III.
 - (i) Whether Claims 119-127, 129-132 and 134-138 are patentable under 35 U.S.C. §102(b) over WO00/18904, (published June/2000); WO99/63088, (published September/1999); WO00/00610, (published June/2000); WO00/00506, (published June/2000).
 - (ii) Whether Claims 119-125, 127, 129-138 are patentable under 35 U.S.C. §102(a) over EP1130094, (published September/2001).

7. ARGUMENT

Summary of the Arguments:

Issue I: Priority

The instant application has not been granted the earlier priority date on the grounds that "the MLR assay fails to provide a specific and substantial utility or well established utility for the claimed invention to satisfy the requirements under 35 U.S.C. § 101/112, first paragraph, because it does not teach how one of ordinary skill in the art could use the claimed invention." (Page 3 of the Final Office Action mailed November 24, 2004).

Appellants submit that data derived from the mixed leukocyte reaction (MLR) assay was first disclosed in U. S. Application Serial No. 60/144,758 filed July 20, 1999 for the claimed PRO1375 polypeptides. Appellants further submit that, the disclosure of U. S. Application Serial No. 60/144,758 is sufficient to establish patentable utility for the claimed polypeptides for the same reasons discussed below under the section on Issue II: Utility/ Enablement. The currently pending claims are therefore entitled to the July 20, 1999, priority date of U.S. Provisional Application No. 60/144,758 in which the results of the MLR assay were first disclosed.

Issue II: Utility/ Enablement

Appellants submit that patentable utility for the nucleic acids encoding the PRO1375 polypeptide is based upon the data derived from the mixed leukocyte reaction (MLR) assay. Example 151 shows that PRO1375 tested positive in the mixed lymphocyte reaction (MLR) assay, demonstrating that PRO1375 is active as a stimulator of the proliferation of stimulated T-lymphocytes, and therefore has utility in the treatment of conditions where the enhancement of an immune response would be beneficial. At the priority date of the present application it was well known that stimulators of T-cell proliferation find utility in fighting viral infections, including retroviral infections, such as HIV infection or Epstein-Barr infection, as well as in the treatment of cancers such as melanoma.

Appellants have also submitted with their Response filed August 5, 2004, the Declaration by Dr. Sherman Fong, a unquestionable expert in the field, which provides examples of important clinical applications for immune stimulants which have shown activity in a mixed lymphocyte reaction assay, such as the chemokine IL-12, which finds utility in the treatment of

melanoma due to its ability to stimulate immune response. Dr. Fong states that "a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant."

Despite Dr. Fong's unequivocal statement and the teachings of the present application, the Examiner has asserted that "the MLR is an *in vitro* model for allogenic reaction, this assay has not been correlated with a specific disease or disorder.... MLR is typically used for determining histocompatibility in an individual and as a test for immunocompetence of T cells in patients with immunodeficiency disorders." In support of this assertion, the Examiner has cited references by Kahan *et al.*, Picotti *et al.* and Campo *et al.* The Examiner also takes issue with Dr. Fong's declaration and quotes Picotti *et al.* and Nishioka *et al.* to show that "the fact that an agent stimulates or inhibits T-cells in the MLR assay, is not sufficient to explain the role of said agent in the immune system." (Page 12, Final Office Action mailed November 15, 2004).

First of all, the evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration. Thus, to overcome the presumption of truth that an assertion of utility by the Appellant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner has made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the Appellant.

The Examiner has not shown that a lack of correlation typically exists between the results of the MLR assay *in vitro* and immunomodulatory activity *in vivo* by quoting the references Kahan *et al.*, Picotti *et al.*, Campo *et al.* and Nishioka *et al.*. In fact, Appellants submit that the teachings within Picotti *et al.*, Campo *et al.* and Nishioka *et al.* support the Appellants' position that the *in vitro* MLR assay can and has been successfully used to identify compounds having immunomodulatory activity in vivo. Therefore, based on such teachings, one of ordinary skill in the art would find it 'more likely than not' that those molecules which test positive in the disclosed MLR assay would have real-world therapeutic utility as immunostimulants *in vivo*; that is, how to use the claimed polypeptides for the treatment of viral infections and cancer, without any undue experimentation. Thus the Patent Office has failed to meet its initial burden of proof

that Appellants' claims of utility are not substantial or credible.

Appellants add that the Examiner has misinterpreted the focus of the assay disclosed in the specification. The purpose of the assay is not to test the properties of the stimulator cells, as in a typical MLR assay, but to test the ability of a protein, such as PRO1375, to enhance the expected response of the T cells, thus demonstrating general immunostimulant activity.

Regarding the enablement rejection, Appellants note that the claimed variants, in addition to having at least 80% nucleic acid sequence identity to SEQ ID NO:417, also have the functional recitation that "said polypeptide induces proliferation of stimulated T lymphocytes in a mixed lymphocyte reaction." Thus the claimed variants all share the disclosed utility of the PRO1375 polypeptide as an immunostimulant. The specification provides ample guidance to allow the skilled artisan to identify those nucleic acids encoding the polypeptide variants which meet the recitations of the claims, including a detailed protocol for the MLR assay. Accordingly, one of ordinary skill in the art would understand how to use the recited the nucleic acids encoding the polypeptide variants without any undue experimentation.

Accordingly, Appellants submit that the MLR assay suffices to provide patentable utility for the subject matter of the instant claims and that, in view of this, one of skill in the art would know exactly how to use the polypeptides encoded for by the claimed nucleic acids, for instance, in the treatment of viral infections or cancer, without any undue experimentation.

Issue III: Anticipation by WO00/18904, WO99/63088, WO00/00610, WO00/00506 and further, anticipation by EP1130094

(i) Claims 119-127, 129-132 and 134-138 stand rejected under 35 U.S.C. §102(a) as being anticipated by WO00/18904, (published June/2000); WO99/63088, (published September/1999); WO00/00610, (published June/2000); WO00/00506, (published June/2000).

(ii) Claims 119-127, 129-132 and 134-138 stand further rejected under 35 U.S.C. §102(a) as being anticipated by EP1130094, (published September/2001).

The instant application claims priority to U.S. Provisional Application Serial No. 60/144,758, filed on July 20, 1999, over six months before the publication date of WO00/18904, WO00/00610, WO00/00506, and at least one month before the publication date of WO99/63088. U.S. Provisional Application Serial No. 60/144,758 first disclosed that the PRO1375

polypeptide tested positive in the MLR assay. Further, since the instant application is entitled to priority to U.S. Provisional Application Serial No. 60/144,758, and to the effective filing date of July 20, 1999, EP1130094 is not prior art.

These arguments are all discussed in further detail below under the appropriate headings.

Detailed Arguments:

ISSUE I: U.S. Provisional Application No. 60/144,758 Satisfies the Utility Requirement of 35 U.S.C. § 101/ § 112, First Paragraph based on the results of the MLR assay

Appellants have asserted that U.S. Provisional Application No. 60/144,758, filed July 20, 1999, discloses stimulatory activity in the mixed leukocyte reaction (MLR) assay, and that the data generated in the MLR assay (shown in Example 151 of the instant specification) establishes patentable utility for the claimed nucleic acids encoding PRO1375 polypeptides. The Examiner has asserted that "(i)t is not disputed that US Provisional application 60/144,758 filed 20 July 1999 discloses the MLR assay. However, the MLR assay fails to provide a specific and substantial utility or well established utility for the claimed invention to satisfy the requirements under 35 U.S.C. § 101/112, first paragraph, because it does not teach how one of ordinary skill in the art could use the claimed invention." (Page 3 of the Final Office Action mailed November 15, 2004).

Appellants strongly disagree and submit that, for the same detailed reasons set forth below under Issue II for Utility/ Enablement (whose arguments are incorporated by reference herein), the results of the MLR assay for PRO1375 in the specification of the provisional U.S. Application No. 60/144,758, provides at least one credible, substantial and specific asserted utility for the claimed PRO1375 polypeptides under 35 U.S.C. §101/§112, first paragraph. Accordingly, Appellants submit that the subject matter of the instant claims should be entitled to an effective priority date of July 20, 1999, the filing date of U.S. Provisional Application No. 60/144,758.

ISSUE II: The Data Generated in the MLR Assay Satisfies the Utility Requirement of 35

U.S.C. § 101/ § 112, First Paragraph for Claims 119-127 and 129-131

Appellants submit that the results of the MLR assay in the instant specification (and in the priority provisional application No. 60/144,758) provides at least one credible, substantial and specific asserted utility for the claimed nucleic acids encoding PRO1375 polypeptides under 35 U.S.C. §101/§112, first paragraph.

A. The Legal Standard for Utility

According to 35 U.S.C. § 101:

Whoever invents or discovers any new and *useful* process, machine, manufacture, or composition of matter, or any new and *useful* improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title. (Emphasis added.)

In interpreting the utility requirement, in *Brenner v. Manson*¹ the Supreme Court held that the *quid pro quo* contemplated by the U.S. Constitution between the public interest and the interest of the inventors required that a patent applicant disclose a "substantial utility" for his or her invention, i.e. a utility "where specific benefit exists in currently available form."² The Court concluded that "a patent is not a hunting license. It is not a reward for the search, but compensation for its successful conclusion. A patent system must be related to the world of commerce rather than the realm of philosophy."³

Later, in *Nelson v. Bowler*⁴ the C.C.P.A. acknowledged that tests evidencing pharmacological activity of a compound may establish practical utility, even though they may not establish a specific therapeutic use. The court held that "since it is crucial to provide researchers

¹ *Brenner v. Manson*, 383 U.S. 519, 148 U.S.P.Q. (BNA) 689 (1966).

² *Id.* at 534, 148 U.S.P.Q. (BNA) at 695.

³ *Id.* at 536, 148 U.S.P.Q. (BNA) at 696.

⁴ *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. (BNA) 881 (C.C.P.A. 1980).

with an incentive to disclose pharmaceutical activities in as many compounds as possible, we conclude adequate proof of any such activity constitutes a showing of practical utility."⁵

In *Cross v. Iizuka*⁶ the C.A.F.C. reaffirmed *Nelson*, and added that *in vitro* results might be sufficient to support practical utility, explaining that "*in vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with the particular pharmacological activity are generally predictive of *in vivo* test results, i.e. there is a reasonable correlation there between."⁷ The court perceived "No insurmountable difficulty" in finding that, under appropriate circumstances, "*in vitro* testing, may establish a practical utility."⁸

Furthermore, M.P.E.P. 2107.03 (III) states that:

"If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process."

Thus, the legal standard accepts that *in vitro* or animal model data is acceptable utility as long as the data is "reasonably correlated" to the pharmacological utility described.

The case law has also clearly established that applicants' statements of utility are usually sufficient, unless such statement of utility is unbelievable on its face.⁹ The PTO has the initial burden to prove that applicants' claims of usefulness are not believable on their face.¹⁰ In general, an Applicant's assertion of utility creates a presumption of utility that will be sufficient

⁵ *Id.* at 856, 206 U.S.P.Q. (BNA) at 883.

⁶ *Cross v. Iizuka*, 753 F.2d 1047, 224 U.S.P.Q. (BNA) 739 (Fed. Cir. 1985).

⁷ *Id.* at 1050, 224 U.S.P.Q. (BNA) at 747.

⁸ *Id.*

⁹ *In re Gazave*, 379 F.2d 973, 154 U.S.P.Q. (BNA) 92 (C.C.P.A. 1967).

¹⁰ *Ibid.*

to satisfy the utility requirement of 35 U.S.C. §101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope."^{11, 12}

Compliance with 35 U.S.C. §101 is a question of fact.¹³ The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration.¹⁴ Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence.

The well established case law is clearly reflected in the Utility Examination Guidelines ("Utility Guidelines")¹⁵, which acknowledge that an invention complies with the utility requirement of 35 U.S.C. §101, if it has at least one asserted "specific, substantial, and credible utility" or a "well-established utility." Under the Utility Guidelines, a utility is "specific" when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic without also identifying the conditions that are to be diagnosed.

In explaining the "substantial utility" standard, M.P.E.P. §2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. "Rather, any reasonable use that an applicant has identified for the invention that

¹¹ *In re Langer*, 503 F.2d 1380, 1391, 183 U.S.P.Q. (BNA) 288, 297 (C.C.P.A. 1974).

¹² See also *In re Jolles*, 628 F.2d 1322, 206 U.S.P.Q. 885 (C.C.P.A. 1980); *In re Irons*, 340 F.2d 974, 144 U.S.P.Q. 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 U.S.P.Q. 209, 212-13 (C.C.P.A. 1977).

¹³ *Raytheon v. Roper*, 724 F.2d 951, 956, 220 U.S.P.Q. (BNA) 592, 596 (Fed. Cir. 1983) cert. denied, 469 US 835 (1984).

¹⁴ *In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d (BNA) 1443, 1444 (Fed. Cir. 1992).

¹⁵ 66 Fed. Reg. 1092 (2001).

can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a ‘substantial’ utility.”¹⁶ Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement,¹⁷ gives the following instruction to patent examiners: “If the applicant has asserted that the claimed invention is useful for any particular practical purpose . . . and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.”

B. Proper Application of the Legal Standard

Appellants submit that the mixed lymphocyte reaction (MLR) assay described in Example 151 is sufficient to establish patentable utility under 35 U.S.C. §101 for the PRO1375 polypeptide and nucleic acids encoding it. The positive result for PRO1375 in the MLR assay, described in Example 151, at page 525 of the specification, demonstrates that PRO1375 is active as a stimulator of the proliferation of stimulated T-lymphocytes.

The MLR is a well-established assay for evaluating test compounds, such as the PRO1375 polypeptide, for their ability to stimulate T-lymphocyte proliferation *in vitro*, and consequently, for assessing the immune response of an individual. The MLR assay is well-described in standard textbooks, including, for example, *Current Protocols in Immunology*, unit 3.12; edited by J E Coligan, A M Kruisbeek, D H Marglies, E M Shevach, W Strober, National Institutes of Health, Published by John Wiley & Sons, Inc., which is referenced in Example 74, and the entire content of which is expressly incorporated by reference into the disclosure of the present application (see page 525). In brief, in this method, an immune response results upon mixing T-cells from antigenically distinct individuals under cell culture conditions. An MLR reaction can be monitored quantitatively by, for example, following the incorporation of tritiated thymidine during DNA synthesis, or by observing blast formation, or by other methods well known in the art.

According to the specification, positive increases over control in this assay are considered to be positive results, with increases of greater than or equal to 180% being preferred. However, any

¹⁶ M.P.E.P. §2107.01.

¹⁷ M.P.E.P. §2107 II (B)(1).

value greater than control indicates a stimulatory effect for the test protein. PRO1375 (SEQ ID NO: 418) tested positive in this assay, using the described criteria. Example 151 further explains that compounds which stimulate proliferation of lymphocytes in this assay "are useful therapeutically where enhancement of an immune response is beneficial." Accordingly, PRO1375 has utility in the treatment of conditions where the stimulation of lymphocyte proliferation would be desirable. Accordingly, one skilled in the art would know how to use the polypeptides encoded for by the claimed nucleic acids, for instance, in the treatment of viral infections or cancer, without any undue experimentation.

In support of utility based upon the MLR assay, Appellants have submitted (with their Response filed August 5, 2004) the Declaration of Sherman Fong, Ph.D. Dr. Fong is an inventor of the above-identified patent application, and an experienced scientist familiar with the MLR assay, which was used by him and others under his supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project, including PRO1375.

The Fong Declaration explains how the MLR reaction was performed in the instant application using peripheral blood mononuclear cells (PBMCs), which contain responder T-cells, and allogenic, pre-treated (irradiated) PBMCs, which predominantly contained dendritic cells. Dr. Fong proceeds to explain (paragraph 7 of the Declaration) that dendritic cells are potent antigen-presenting cells that are able to "prime native T cells *in vivo*." Once activated by dendritic cells, the T-cells are capable of interacting with other antigen-presenting cells (B cells and macrophages) to produce additional immune responses from these cells.

As Dr. Fong states, the MLR assay of the present application

is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously. (Paragraph 8 of the Fong Declaration.)

As Dr. Fong emphasizes, immunostimulants are important and highly desirable in the treatment of cancer and in enhancing the effectiveness of previously identified treatments for cancer. Supportive evidence also comes from teachings in the art such as Steinman *et al.* (submitted as Exhibit B with the Preliminary Amendment filed July 12, 2004) who state that

"...medicine needs therapies that enhance immunity or resistance to infections and tumors"
(page 1, column 1, line 7; emphasis added).

In paragraph 9 of his Declaration, Dr. Fong provides examples of important clinical applications for immune stimulants which have been shown to stimulate T-cell proliferation in the MLR assay. As Dr. Fong explains,

"IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured *in vitro* and returned to the patient to stimulate anti-cancer response. [Thurner et al. J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)]."

Dr. Fong concludes that (paragraph 10):

It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant."

Accordingly, the positive results obtained in this assay clearly establish the immunostimulant utility for the PRO1375 polypeptides for instance, in the treatment of viral infections or cancer, without any undue experimentation, and thus, utility for the claimed nucleic acids that encode such polypeptides that are claimed in the present application, for the asserted purpose.

C. A prima facie case of lack of utility has not been established

As a preliminary matter, Appellants submit that, the evidentiary standard to be used throughout *ex parte* examination of a patent application is a preponderance of the totality of the

evidence under consideration. Accordingly, Appellants submit that in order to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that **it is more likely than not** that one of ordinary skill in the art would doubt the truth of the statement of utility. With respect to asserted therapeutic utilities based upon *in vitro* data, an applicant "does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty" (M.P.E.P. 2107.03.). The law requires only that one skilled in the art should accept that such a correlation is **more likely than not to exist**. Appellants respectfully submit that when the proper evidentiary standard is applied, a correlation must be acknowledged.

(i) An *in vitro* assay like the MLR assay is useful for identifying compounds with immunomodulatory activity *in vivo*

The Examiner alleges that the fact situation in Cross *et al.* v. Iizuka *et al.* is different from the fact pattern of the instant case because Cross *et al.* v. Iizuka *et al.* discloses imidazole derivative compounds which inhibit synthesis of thromoxane synthetase, an enzyme involved in platelet aggregation. The Examiner adds that platelet aggregation is associated with several deleterious conditions in mammals such as platelet thrombosis, pulmonary vasconstriction, etc. The Examiner alleges that, on the other hand, the instant case "(t)he MLR is an *in vitro* model for allogenic reaction, this assay has not been correlated with a specific diseases or disorder." (Page 5 of the Final Office Action mailed November 15, 2004). Regarding the Appellants previous assertion that "PRO1375 is active as a stimulator of the proliferation of stimulated T-lymphocytes, and therefore would have utility in the treatment of conditions where the enhancement of an immune response would be beneficial," the Examiner alleges that "(the) instant specification has not established a correlation between the claimed nucleic acid or the encoded protein and any of the above mentioned disorders. The specification has not shown that the protein of the instant invention has been used to treat any of these diseases." Appellants strongly disagree.

First of all, Appellants submit that the fact situation in Cross *et al.* v. Iizuka *et al.* is entirely applicable to the instant case since, and as will be apparent in the discussions below, Appellants assert utility for PRO1375 as an immunostimulant that is useful in treating several

disease conditions where the enhancement of an immune response would be beneficial, including but not limited to, viral infections, including retroviral infections like HIV or Epstein-Barr infections, as well as in the treatment of cancers such as melanoma. Appellants submit that the teachings of the specification should be evaluated through the eyes of one skilled in the pertinent art at the effective filing date of the present application. For instance, in 1998, well before the effective filing date of July 20, 1999 of the instant application, it was well known in the art as it is today that, T-cells were highly important in the body's natural defense mechanisms for fighting infections. For example, viral infections, such as HIV infection, were well known to result in a reduced T cell count. Indeed, the count of T-cell lymphocytes was a generally accepted measure for the extent and seriousness of an HIV infection and resultant AIDS. Accordingly, stimulators of T-cell proliferation were "reasonably correlated" to the art- accepted, pharmacological utility of fighting viral infections, including retroviral infections such as HIV infections or Epstein-Barr infections.

It was also well known at the time of filing that T cells could recognize tumor antigens and kill tumors. See, for example. Thurner *et al.*, J. Exp. Med. 190:1669-1678 (1999) (submitted as Exhibit E with the Fong declaration filed August 5, 2004), which describes experimental procedures designed to treat melanoma by boosting the immune response. Accordingly, immunostimulant molecules such as PRO1375 would have an art-accepted utility in the treatment of cancers such as melanoma.

Appellants further submit that the legal standard accepts *in vitro* results as a demonstration of utility, as long as the data is "reasonably correlated" to the pharmacological utility described (for details, see Legal Standard above). For instance, M.P.E.P. 2107.03 (III) states that:

"If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process."

Thus, the data derived from the MLR assay is "reasonably correlated" to at least one asserted pharmacological utility in diseases such as, in the treatment of HIV or Epstein Barr infections or in the destruction of tumors in various cancers, and meets the utility standards. The art

recognizes that the MLR assay is an *in vitro* assay useful for identifying compounds with immunomodulatory activity *in vivo*.

(ii) The art in fact supports the Appellants' position that an MLR result is useful for identifying compounds with immunomodulatory activity in vivo

The Examiner asserts on Page 5 that "the state of the art is such that no *in-vitro* immune assay predicts or correlates with *in vivo* immunosuppressive efficiency". In support of these assertions, the Examiner has cited references by Kahan *et al.*, Picotti *et al.* and Campo *et al.*

Appellants respectfully disagree. All three references, Kahan *et al.*, Picotti *et al.* and Campo *et al.*, study allograft rejections and immunosuppression of graft rejection using test compounds studied *in vitro*. Appellants submit that the Examiner has not correctly characterized the teachings of Kahan *et al.*, Picotti *et al.* and Campo *et al.*. These references, in combination with those cited by Appellants, in fact demonstrate that the art as a whole recognizes that the mixed lymphocyte reaction (MLR) is in fact a widely used *in vitro* assay for identifying immunomodulatory compounds.

For instance, the Examiner cited Picotti *et al.* as demonstrating that "IL-12 enhances alloantigen-specific immune function as determined by MLC, but this result *in vitro* does not result in a measurable response *in vivo* (i.e. failure to accelerate graft rejection) see p 1459."

Appellants note that the asserted utility of PRO1375 is not to accelerate allograft rejection, which is hardly a process that medical practitioners would wish to accelerate. Thus the fact that IL-12 does not accelerate allograft rejection *in vivo* is not relevant to the utility of PRO1375. More importantly, Appellants draw the Board's attention to the fact that the failure of IL-12 to accelerate allograft rejection *in vivo* is not necessarily an indication that IL-12 does not possess general immunostimulant properties *in vivo*. For example, Picotti *et al.* confirm that "IL-12 is also a key cytokine involved in promoting cell mediated immune responses *in vivo*" (page 1459, col. 1). Thus, the fact that a molecule such as IL-12, which is a known immunostimulant *in vivo*, does not accelerate graft rejection supports Appellants' argument that graft rejection is a specific pathway that does not necessarily reflect general immunoregulatory function. Picotti *et al.* too draw a similar conclusion, suggesting that "the magnitude of Th1-driven alloimmune response may not correlate directly to the severity of graft rejection," perhaps because Th2-driven

immune responses are more relevant to graft rejection (page 1459, col. 2). Therefore, Picotti *et al.*, provides evidence that a molecule which does not show *in vivo* activity in a specific graft versus host interactive pathway may still have general immunomodulatory activity.

The Examiner has further asserted that Campo *et al.* "demonstrate that while zinc suppresses alloreactivity in MLC, it does not decrease T-cell proliferation *in vitro* nor produce immunosuppressive effects *in vivo*." (Page 6 of the Final Office Action mailed November 15, 2004).

Appellants respectfully point out that the Examiner has misinterpreted this statement, due to the fact that the authors refer to two different types of immunosuppressive effects. Campo *et al.* set out to look for an inhibitor of MHC *in vitro* which would have the fewest side effects *in vivo* (see Abstract). The authors note that high concentrations of zinc "impair **all** T cell and monocyte function" (page 20; emphasis added). The authors took this impairment as an indicator of toxicity, and therefore intentionally used concentrations of zinc below that at which all T-cell function was impaired, in order to identify a concentration range that would not result in toxic effects. However, that does not mean that Campo *et al.* found zinc to have no immunosuppressive activity *in vivo*. In fact, the authors conclude, based upon their MLC results, that "zinc could become an immunosuppressant in transplantation medicine without toxic side effects" (page 21; emphasis added). Thus Campo *et al.* supports Appellants' position that those of skill in the art would interpret the results of MLC assays as having physiological relevance.

In fact, Appellants note that the Examiner has failed to point out several instances within these cited references wherein the authors stated that the MLR is an important method with a good predictive value. For example, Campo *et al.* teach that "the human mixed lymphocyte culture (MLC) is an important method to test donor-recipient compatibility in bone marrow transplantation. It could be shown that cytokine release, especially IFN- γ , **has a very good predictive value with regard to the transplantation outcome**, as cytokines play a major role in the generation of an alloreactive immune response and for the induction of graft rejection *in vivo*.....Landolfo *et al.* inhibited T-cell reactivity by the addition of anti-IFN- γ **both *in vitro* and *in vivo***" (see page 18; emphasis added). Further, Picotti *et al.* showed that the IL-12R β 1 subunit was critical for IL-12 driven enhanced alloimmune response *in vitro* and *in vivo* (see abstract). Thus, while there are instances of unpredictability using the MLR assay, there are many studies

showing predictable results, including studies from Picotti, Landolfo and the IFN- γ study. Finally, Campo *et al.* teaches that "cyclosporin A, FK506, and other substances are used to prevent graft rejection. **In vitro experiments revealed an inhibition of the MLC**" (page 16). Thus the teachings of Campo *et al.* confirm that inhibition of the MLR is observed for known immunoinhibitory molecules, that are in actual clinical use.

Thus, in fact, references Picotti *et al.* and Campo *et al.* support the Appellants' position that it is more likely than not that the *in vitro* MLR assay can be successfully used to identify compounds having immunomodulatory activity *in vivo*.

(iii) The specification provides sufficient detail to enable one of ordinary skill in the art to conclude that the instant MLR assay is useful to identify compounds having immunomodulatory activity in vivo

The Examiner has further asserted on Page 7, line 4 of the Final Office Action that "(t)he specification fails to provide any data or evidence of the results of the assay, therefore, one of ordinary skill in the art cannot evaluate the conclusion."

Appellants respectfully submit that the specification clearly discloses that PRO1375 tested positive in the MLR assay. Further, the teachings of the specification should be evaluated through the eyes of one skilled in the pertinent art at the effective filing date of the present application. For instance, in 1998, well before the effective filing date of July 20, 1999 of the instant application, it was well known in the art as it is today that, T-cells were highly important in the body's natural defense mechanisms for fighting infections. For example, viral infections, such as HIV infection, were well known to result in a reduced T cell count. It was also well known at the time of filing that T cells could recognize tumor antigens and kill tumors. The Fong Declaration reinforces the teachings of the specification that a PRO polypeptide with an activity in the MLR assay of at least 180% of the control is expected to have the type of activity exhibited by IL-12, and would therefore find practical utility as an immune stimulant. Therefore, one of ordinary skill in the art would understand that any molecule showing immunostimulant activity as measured in the MLR assay, would be useful in the treatment of conditions where the stimulation of lymphocyte proliferation would be desirable, including viral infections such as

HIV and Epstein-Barr, and cancers such as melanoma based on references cited in the art, including the Examiner cited references like Picotti *et al.* and Campo *et al.*

The Examiner also makes detailed arguments regarding the instant MLR controls and MLR's use in only determining histocompatibility. On Page 6, second paragraph, the Examiner says, "MLR is typically used for determining histocompatibility in an individual and as a test for immunocompetence of T cells in patients with immunodeficiency disorders,"

Appellants note that the MLR assay also depends upon the interactions of T lymphocytes with other cells, in particular, with the stimulator cells used in the described MLR protocol, as explained in the Fong declaration, and as shown in MLR assays performed within the Examiner cited references like Picotti *et al.* and Nishioka *et al.* T cells cooperate with antigen presenting cells like dendritic cells *in vivo*, which are the most potent antigen presenting cells, and are the predominant cell type found in the irradiated stimulator cell population as explained in the Fong Declaration (see paragraphs 5-6). Thus the MLR assay can and does measure the interaction of T-lymphocytes with other cells as well, and does not only measure histocompatibility of individuals.

Further, Appellants add that the Examiner appears to have misinterpreted the intent of the MLR assay in the instant application. The mixing of the stimulator and responder cells in the instant MLR is expected to lead to T cell proliferation even in the absence of any test protein. However, the point of the assay is to measure the extent to which the test protein can enhance the expected proliferation of the stimulated T cells.

The Examiner's misunderstanding of the instant MLR assay is further demonstrated in the Examiner's discussion of "controls" (page 6-7, last paragraph onwards of the Final Office Action mailed November 15, 2004). The Examiner has asserted that "(t)here are several controls which the art recognizes as being essential for meaningful results for this assay, including autologous controls, a control to determine maximum response, screening for possible HLA antibodies and growth support capabilities. There is known inherent variability of individual cellular responses from day to day, which would clearly dictate the need for internal controls."

Appellants submit that these controls are only needed when the purpose of carrying out the MLR assay is to evaluate the properties of the stimulator cells. The purpose of the assay disclosed in the instant specification, as discussed above, is to characterize, not the stimulator

cells, but the test proteins, such as PRO1375. The precise extent to which the stimulator cells stimulate the responder cells is not significant; what matters is the degree to which the test protein increases this response. The extent to which the test protein increases the response of the T cells is measured by comparison to a negative control reaction, which uses either cell culture medium, or a non immunostimulant molecule, CD4-IgG, as a negative control. Because the response in the test reaction is compared to a negative control reaction, and because both reactions use the same stimulator and responder cells at the same time, additional controls to determine the precise properties of these cells are not required.

In summary, Appellants respectfully submit that the Examiner has not shown that a lack of correlation typically exists between results of the MLR assay *in vitro* and immunomodulatory activity *in vivo*. In fact, references Picotti *et al.* and Campo *et al.* support the Appellants' position that it is more likely than not that the *in vitro* MLR assay can be successfully used to identify compounds having immunomodulatory activity *in vivo*. Therefore, the Patent Office has failed to meet its initial burden of proof that Appellants' claims of utility are not substantial or credible.

D. The Fong Declaration supports "real world" utility for proteins that test positive in the MLR assay

The Examiner asserted that "Dr. Fong's Declaration is fully considered, but is not effective to overcome the rejection....under 35 U.S.C. 101/112, first paragraph".

Appellants strongly disagree. The case law has clearly established that in considering affidavit evidence, the Examiner must consider all of the evidence of record anew.¹⁸ "After evidence or argument is submitted by the Appellant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument"¹⁹ Furthermore, the Federal Court of Appeals held in *In re Alton*, "We are aware of no reason why opinion evidence relating to a fact issue should not be

¹⁸ *In re Rinehart*, 531 F.2d 1084, 189 U.S.P.Q. 143 (C.C.P.A. 1976) and *In re Piasecki*, 745 F.2d. 1015, 226 U.S.P.Q. 881 (Fed. Cir. 1985).

¹⁹ *In re Alton*, 37 U.S.P.Q.2d 1578 (Fed. Cir. 1966) at 1584 quoting *In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992)).

considered by an examiner"²⁰. Appellants also respectfully draw the Board's attention to the Utility Examination Guidelines²¹ which state, "Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered." Thus, barring evidence to the contrary, Appellants maintain that the positive result for PRO1375 in the MLR assay is significant, and forms the basis for the utility claimed herein.

Further, the Examiner adds that "the MLR assay in itself is not predictive of an *in vivo* immune response, as was shown by Picotti *et al.*" While the Examiner acknowledges on page 11, line 1 of the Final Office action that "IL-12 is a known immune stimulator, which was first identified in an MLR assay," the Examiner later adds on page 11, line 19 that, "there are discrepancies of the effect of IL-2 on MLR assay depending on what stimulator is used." The Examiner cites Nishioka *et al.* as stating "IL-12 effects differentially in human or mice cellular immunity in MLR stimulated by dendritic cells. Although IL-12 is shown to suppress MLR in mice, IL-12 stimulates MLR in humans." Thus, the Examiner concludes that "the effect of IL-12 in the MLR assay alone was not sufficient to elucidate the role of this cytokine in the immune system." (Page 12 of the Final Office Action mailed November 15, 2004).

As discussed above, Picotti *et al.* in fact provides supportive evidence and confirms that that a given molecule (like IL-12) that may not accelerate allograft rejection *in vivo* may still possess properties of a general immunostimulant in cell mediated immune responses *in vivo* (see page 1459, col.1). Therefore, Picotti *et al.* is not an appropriate reference for rejecting the Fong declaration.

Moreover, regarding the rejection of the Fong declaration based on Nishioka *et al.*, Appellants submit that the Nishioka *et al.* also provides supportive evidence for the Appellants' position that the art as a whole recognizes that the mixed lymphocyte reaction (MLR) is in fact a widely used *in vitro* assay for identifying immunomodulatory compounds. For instance, Nishioka *et al.* themselves provide compelling reasons for the observed discrepancies that the

²⁰ *In re Alton, supra.*

²¹ Part IIB, 66 Fed. Reg. 1098 (2001).

Examiner refers to in the rejection. The authors say on page 627, first column, second paragraph, line 10 that:

"(t)hese discrepancies between the previous reports and ours might be a result of the differences of stimulator used in MLR. BM-DCs (bone marrow derived dendritic cells) were used in our experiments and splenocytes in the other as a stimulator. Splenocytes contain a DC population, but the percentage of DCs in the spleen is 1.0-1.6%. DCs are stronger stimulators in MLR when compared with splenocytes. Therefore, the stimulation by DCs might lead to a higher production of NO and the subsequent suppression of MLR."

The authors further acknowledged on page 626, in the second paragraph of the discussion section, that:

"IL-12 is known to play an important role in promoting Th1 differentiation and cellular immune responses. The numerous studies demonstrated the antitumor effects of IL-12, whereas the detailed mechanisms remained unclear as a result of the complex effects of IL-12" (emphasis added).

The authors add on the last line of the discussion on page 627 that:

"(a)s NO induced by IL-12 *in vivo* is likely to be less in humans, **the antitumor effect mediated by IL-12 via cellular immune responses might be expected in humans rather than in mice**" (emphasis added).

The authors conclude on the last line of the abstract that:

"(t)hese results suggest that NO produced by DCs (dendritic cells) might play an important role in IL-12 mediated immune suppression in mice but not in humans."

Therefore, Appellants submit that the Examiner has not made a compelling case for rejecting the Fong Declaration based on the teachings of Picotti *et al.* or Nishioka *et al.*, because these references, in fact, support the Appellants' position that results from an MLR assay indicate an immunomodulatory activity for that molecule *in vivo*. Even if discrepancies between MLR studies by various groups may occur, a careful analysis of the materials used in the assay, for instance, the type of immune cells used like DCs (dendritic cells) versus splenocytes, mouse cells versus human cells, etc., can elucidate the reasons underlying the mechanism for the discrepancy as explained in Nishioka *et al.* Although such experiments further the understanding of the immunomodulatory mechanism, they are confirmatory studies, and would not be considered

undue by the skilled artisan. Moreover, they are not essential for utility determination of the molecule. Therefore, Appellants submit that this rejection is improper under both the case law and the Utility guidelines.

E. One Skilled in the Art would know how to make and use the variant proteins without undue experimentation based on the teachings in the art and in the specification

The Examiner had rejected the instant claims under 35 U.S.C. 112, first paragraph because "although the claims recite both percent identity and functional language, the specification does not disclose a variant of the polypeptide of SEQ ID NO:418 that induces proliferation of stimulated T lymphocytes in a MLR. Due to the large quantity of experimentation necessary to determine all the nucleic acids comprising a nucleotide sequence that is at least 80%, 85%, 90%, 95% or 99% identical to the polypeptide of SEQ ID NO:418 and to screen for the ones that induce proliferation of stimulated T lymphocytes in a MLR, the lack of direction/ guidance..., the complex nature of the invention, the absence of working examples..., the state of the prior art establishing that biological activity cannot be predicted based on structural similarity, the unpredictability of the effects of mutation on the structure and function of the claimed polypeptide, the breadth of the claims which fail to recite particular biological activities, undue experimentation would be required...." (Page 8, line 8 onwards of the Final Office Action mailed November 15, 2004).

Appellants respectfully disagree. As the M.P.E.P. states, "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation."²² As discussed above, a considerable amount of experimentation is permissible, if it is merely routine.

Appellants refer to the arguments presented above with respect to utility and these arguments are incorporated by reference herein. Appellants note that the claimed nucleic acid variants all share the functional recitation that "wherein the polypeptide encoded by said nucleic acid induces proliferation of stimulated T lymphocytes in a mixed lymphocyte reaction." Example 151 of the present application provides detailed protocols for the MLR assay, including

²² M.P.E.P. §2164.01 citing *In re Certain Limited-charge Cell Culture Microcarriers*, 221 U.S.P.Q. 1165, 1174 (Int'l Trade Comm'n 1983), *aff' sub nom. Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 U.S.P.Q. 428 (Fed. Cir. 1985).

the extensive step-by-step guidance from Current Protocols in Immunology, which is explicitly incorporated into the specification by reference. By following the disclosure in the specification, one skilled in the art can easily test whether a PRO1375 polypeptide, encoded by any variant nucleic acid claimed herein, is capable of stimulating proliferation of T-lymphocytes. Appellants recognize that there may be polypeptides, encoded by such variant nucleic acids, that are (i) structurally related to PRO1375 but which do not test positive in the MLR assay or, (ii) do not resemble PRO1375 in structure but stimulate the immune system through mechanisms unrelated to those of PRO1375. Such variant nucleic acids encoding the PRO1375 polypeptides described above however, would not be encompassed by the instant claims because Appellants claim only those variant nucleic acids that meet the structural requirements defined in the claims and whose encoding proteins meet the functional requirements defined in the claims. Thus, these recitations clearly act to further define the claimed genus of variant nucleic acids and this would be clearly evident to one of skill in the art.

The specification further describes methods for the determination of percent identity between two nucleic acid sequences. (See page 309-311, line 22). In fact, the specification teaches specific parameters to be associated with the term "percent identity" as applied to the present invention. Once such a nucleic acid sequence is identified, the specification sets forth methods for making the nucleic acid sequence (see page 309 and Example 135, page 511 of the specification), methods of preparing the corresponding PRO polypeptides (see page 375, line 11 and onward) and detailed guidance as to the changes that may be made to a PRO polypeptide without adversely affecting its activity (page 371, line 6, to page 373, line 17). This guidance includes a listing of exemplary and preferred substitutions for each of the twenty naturally occurring amino acids (Table 6, page 372). Accordingly, one of skill in the art could identify whether a variant nucleic acid sequence coding for a polypeptide of PRO1375 sequence falls within the parameters of the claimed invention.

Moreover, Appellants note that biological activity in this instance is not claimed based on structural similarity. Neither is structure prediction of the claimed variants a requirement for utility of the claimed polypeptides. As discussed above, Appellants claim only those nucleic acids which meet both recitations of the claims, structural and functional. Therefore the effects of mutations on structure-prediction of the variants and the unpredictability in the art are irrelevant to the instant

case. Finally, the breadth of the claims are clearly defined by both the structural and functional recitations.

Therefore, Appellants respectfully submit that the specification provides ample guidance such that one of skill in the art could readily test a variant nucleic acid to determine whether it's encoding polypeptide is capable of stimulating proliferation of T-lymphocytes by the methods set forth in Example 151. One of skill in the art would have understood at the time of filing, based on the design of the instant MLR assay (which is designed to find molecules that increase DC (dendritic cell)-induced T-cell proliferation) and based solely upon the knowledge about DCs and their role in antigen presentation which was widely known that: (i) molecules which enhanced the proliferation of stimulated T-cells would increase the ability of DCs to convert antigens to immunogens, and (ii) such stimulatory molecules would allow antigens that were not usually immunogenic, such as the melanoma or viral antigens described above, to become immunogenic without undue experimentation. Thus, this biological activity together with the well defined relatively high degree of sequence identity and general knowledge in the art at the time the invention was made, sufficiently defines the claimed genus such that, one skilled in the art, at the effective date of the present application, would have known how to make and use the claimed nucleic acid sequences without undue experimentation.

Accordingly, Appellants respectfully request reconsideration and reversal of the enablement rejection of Claims 119-127, 129-132 and 134-138 under 35 U.S.C. §112, first paragraph.

ISSUE III: Claims 119-127, 129-132 and 134-138 are not anticipated under 35 U.S.C. §102(b) by WO00/18904, WO99/63088, WO00/00610, WO00/00506 and further, are not anticipated under 102(a) by EP1130094

(i) Claims 119-127, 129-132 and 134-138 stand rejected under 35 U.S.C. §102(b) as allegedly being anticipated by (b) over WO00/18904, (published June/2000); WO99/63088, (published September/1999); WO00/00610, (published June/2000); WO00/00506, (published June/2000).

Appellants submit that, as discussed above regarding the utility and enablement requirements of 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph, (Issue II), the results of the MLR assay (Example 151) establishes a credible, substantial and specific asserted utility for the

claimed PRO1375 polypeptides. These results were first disclosed in U.S. Provisional Application Serial No. 60/144,758, filed on July 20, 1999 (discussed in Issue I). The disclosure of the instant application, which is similar to that of the earlier-filed application (U.S. Provisional Application Serial No. 60/144,758), provides the support required under 35 U.S.C. §112 for the subject matter of the instant claims. These arguments are incorporated by reference herein. Accordingly, Appellants submit that the subject matter of the instant claims is disclosed in the manner provided by 35 U.S.C. §112 in U.S. Provisional Application Serial No. 60/144,758. Therefore, the effective filing date of this application is July 20, 1999, the filing date of U.S. Provisional Application Serial No. 60/144,758.

That is, the instant application claims priority to U.S. Provisional Application Serial No. 60/144,758, filed on July 20, 1999, over six months before the publication date of WO00/18904, WO00/00610, WO00/00506, and at least one month before the publication date of WO99/63088, and first disclosed that the PRO1375 polypeptide tested positive in the MLR assay.

Accordingly, Appellants respectfully request reconsideration and reversal of the rejection of Claims 119-127, 129-132 and 134-138 under 35 U.S.C. §102(b) as being anticipated by WO00/18904, WO99/63088, WO00/00610, WO00/00506.

(ii) Claims 119-125, 127 and 129-138 stand rejected under 35 U.S.C. §102(a) as being anticipated by EP1130094, (published September/2001).

As discussed above, under Issue I: priority and Issue II: utility/enablement (the above arguments being incorporated by reference herein), the instant application correctly claims priority to U.S. Provisional Application Serial No. 60/144,758, and therefore, is at least entitled to an effective filing date of **July 20, 1999**. Accordingly, EP1130094 (published September/2001) is not prior art. Therefore, Appellants respectfully request reconsideration and reversal of the rejection of Claims 119-125, 127 and 129-138 under 35 U.S.C. §102(a).

CONCLUSION

For the reasons given above, Appellants submit that the MLR assay disclosed in Example 151 of the specification provides at least one patentable utility for the PRO1375 polypeptides, and for nucleic acids encoding the same as claimed in Claims 119-127, 129-132 and 134-138. Appellants further submit that one of ordinary skill in the art would understand how to use the claimed polypeptides, for example in therapeutic applications where enhancement of an immune response is beneficial, such as in the treatment of viral infections or cancer. Therefore, the corresponding encoding nucleic acids are also useful and Claims 119-127, 129-132 and 134-138 meet the requirements of 35 USC §101 and 35 USC §112, first paragraph. Further, this patentable utility for the instantly claimed nucleic acids was first disclosed in U.S. Provisional Application Serial No. 60/144,758, filed on July 20, 1999, priority to which is properly claimed in the instant application. Accordingly, the instant application has an effective priority date of July 20, 1999, and therefore neither WO00/18904, (published June/2000); WO99/63088, (published September/1999); WO00/00610, (published June/2000); WO00/00506, (published June/2000) nor EP1130094, (published September/2001) are prior art and do not anticipate the claims under 35 USC §102(a) or (b).

Accordingly, reversal of all the rejections of Claims 119-127, 129-132 and 134-138 is respectfully requested.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 08-1641 (referencing Attorney's Docket No. 39780-2730 P1C71).

Respectfully submitted,

Date: January 13, 2006

HELLER EHRLIN LLP
275 Middlefield Road
Menlo Park, California 94025-3506
Telephone: (650) 324-7000
Facsimile: (650) 324-0638
SV 2179828 v1
1/12/06 2:47 PM (39780.2730)

for Leslie M. Mori Leslie Mori (Reg. No. 37,047)
Daphne Reddy (Reg. No. 53,507)

8. CLAIMS APPENDIX

Claims on Appeal

119. An isolated nucleic acid having at least 80% nucleic acid sequence identity to:
- (a) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418;
 - (b) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide;
 - (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO: 418;
 - (d) the nucleic acid sequence of SEQ ID NO: 417;
 - (e) the full-length coding sequence of the nucleic acid sequence of SEQ ID NO: 417; or
 - (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 203115;

wherein the polypeptide encoded by said nucleic acid induces proliferation of stimulated T lymphocytes in a mixed lymphocyte reaction.

120. An isolated nucleic acid of Claim 119 having at least 85% nucleic acid sequence identity to:
- (a) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418;
 - (b) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide;
 - (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO: 418;
 - (d) the nucleic acid sequence of SEQ ID NO: 417;
 - (e) the full-length coding sequence of the nucleic acid sequence of SEQ ID NO: 417; or
 - (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 203115;
- wherein the polypeptide encoded by said nucleic acid induces proliferation of stimulated T lymphocytes in a mixed lymphocyte reaction.

121. An isolated nucleic acid of Claim 119 having at least 90% nucleic acid sequence identity to:

- (a) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418;
- (b) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide;
- (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO: 418;
- (d) the nucleic acid sequence of SEQ ID NO: 417;
- (e) the full-length coding sequence of the nucleic acid sequence of SEQ ID NO: 417; or
- (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 203115;

wherein the polypeptide encoded by said nucleic acid induces proliferation of stimulated T lymphocytes in a mixed lymphocyte reaction.

122. An isolated nucleic acid of Claim 119 having at least 95% nucleic acid sequence identity to:

- (a) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418;
- (b) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide;
- (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO: 418;
- (d) the nucleic acid sequence of SEQ ID NO: 417;
- (e) the full-length coding sequence of the nucleic acid sequence of SEQ ID NO: 417; or
- (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 203115;

wherein the polypeptide encoded by said nucleic acid induces proliferation of stimulated T lymphocytes in a mixed lymphocyte reaction.

123. An isolated nucleic acid of Claim 119 having at least 99% nucleic acid sequence identity to:

- (a) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418;
 - (b) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide;
 - (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO: 418;
 - (d) the nucleic acid sequence of SEQ ID NO: 417;
 - (e) the full-length coding sequence of the nucleic acid sequence of SEQ ID NO: 417; or
 - (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 203115;
- wherein the polypeptide encoded by said nucleic acid induces proliferation of stimulated T lymphocytes in a mixed lymphocyte reaction.

124. An isolated nucleic acid comprising:
- (a) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418;
 - (b) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide;
 - (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO: 418;
 - (d) the nucleic acid sequence of SEQ ID NO: 417;
 - (e) the full-length coding sequence of the nucleic acid sequence of SEQ ID NO: 417; or
 - (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 203115.

125. The isolated nucleic acid of Claim 124 comprising a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418.

126. The isolated nucleic acid of Claim 124 comprising a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide.

127. The isolated nucleic acid of Claim 124 comprising the nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide.

128. Canceled.

129. The isolated nucleic acid of Claim 124 comprising the nucleic acid sequence of SEQ ID NO: 417.

130. The isolated nucleic acid of Claim 124 comprising the full-length coding sequence of the nucleic acid sequence of SEQ ID NO: 417.

131. The isolated nucleic acid of Claim 124 comprising the full-length coding sequence of the cDNA deposited under ATCC accession number 203115.

132. An isolated nucleic acid that hybridizes under stringent conditions to:

- (a) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418;
- (b) a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 418, lacking its associated signal peptide;
- (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of SEQ ID NO: 418;
- (d) the nucleic acid sequence of SEQ ID NO: 417;
- (e) the full-length coding sequence of the nucleic acid sequence of SEQ ID NO: 417; or
- (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 203115;

wherein said stringent conditions employ hybridization using 50% formamide, 5X SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, and washes at 42°C in 0.2X SSC, at 55°C in 50% formamide followed by a high-stringency wash at 55°C in 0.1X SSC, EDTA.

133. Canceled.
134. The isolated nucleic acid of Claim 52 which is at least 10 nucleotides in length.
135. A vector comprising the nucleic acid of Claim 124.
136. The vector of Claim 135, wherein said nucleic acid is operably linked to control sequences recognized by a host cell transformed with the vector.
137. A host cell comprising the vector of Claim 135.
138. The host cell of Claim 137, wherein said cell is a CHO cell, an *E. coli* or a yeast cell.

9. EVIDENCE APPENDIX

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Items 1-4 were made of record by the Examiner in the Final Office Action mailed November 15, 2004.

Item 5 was submitted with Appellants' Response filed August 5, 2004, and noted as considered by the Examiner in the Final Office Action mailed November 15, 2004.

10. RELATED PROCEEDINGS APPENDIX

None.

Immunosuppressive therapy

Barry D. Kahan

The University of Texas Medical School, Houston, Texas, USA

Although Cyclosporin A has improved transplant outcome, its use has serious limitations due to its narrow therapeutic window. New approaches to broaden this window exploit alternative drug formulations, pharmacokinetic profiling and new immunosuppressive agents, such as Rapamycin and Brequinar, which act in a synergistic fashion. There is no evidence to suggest that the pharmacological alternative to Cyclosporin A, FK-506, displays a broader therapeutic window; although it may be tenfold more potent. Similarly, despite the specificity of the IgG2a mouse anti-human CD3 monoclonal antibody, it displays a significant range of clinical side effects, delayed therapeutic action and frequently stimulates generation of human anti-mouse monoclonal antibodies. Recent advances in monoclonal antibody technology seek not only to produce antibodies against determinants involved in alloactivation, but also to 'humanize' the antibodies for reduced side effects. The availability of this array of potential agents highlights the need to develop guidelines for clinical trial methodologies to address the unique needs and demands of organ transplantation.

Current Opinion in Immunology 1992, 4:553-560

Introduction

After thirty years of vigorous but relatively unproductive research, the field of immunosuppressive drugs awakened following the approval of two agents that, in contrast to the non-selective drugs Azathioprine (Aza) and corticosteroids, display relatively specific actions on T cells. One of these, the fungal undecapeptide Cyclosporin A (CsA), not only improved clinical outcomes and broadened the clinical settings in which transplants were successful, but also provided a unique tool for dissecting activation mechanisms leading to lymphokine synthesis. Subsequent approval of the other agent, the IgG2a mouse monoclonal antibody (mAb) OKT-3, heralded the use of reagents that bind selective T-cell surface markers to modulate the immune response. The past decade has witnessed striking progress in the development of new pharmacological agents (Fig. 1). One group inhibits lymphokine biosynthesis, FK-506 or signal transduction, Rapamycin (RAPA). A second group is the nucleotide synthesis Inhibitors; Mizoribine [1] and RS61443; a morpholinoethyl-ester analog of mycophenolic acid (MPA) [2**], block purine salvage pathways with the generation of guanosine monophosphate, and the quinoline carboxylic acid Brequinar (BQR) blocks the *de novo* synthesis of pyrimidines [3**]. A third group,

new mAbs, recognizes specific surface epitopes on T cells and antigen-presenting cells (Fig. 2). Immunosuppressive activity has been documented [4-9] with several mAbs that bind various determinants as shown in Table 1. Prolonged graft survival has also been achieved with antibodies, or preferably their F(ab')₂ fragments, directed towards class I [10] or class II MHC antigens. A refinement of mAb technology is the production of immuno-toxins. Ricin α -chain toxin linked to mouse anti-human CD5 IgG1 mAb has been used by Haverty (personal communication) to treat steroid-resistant graft versus host disease in human bone marrow transplantation. This array of new agents proffers an unprecedented opportunity to design effective, yet minimally toxic, regimens to improve the outcome of transplantation in man.

Limitations of existing immunosuppressive regimens

Currently, clinical regimens are based upon the use of CsA, the immunosuppressant benefits of which are seriously limited by side effects. In attempts to augment its efficacy, the corticosteroid Prednisone (Pred), Aza,

Abbreviations

- ALG—anti-lymphocyte sera; Aza—Azathioprine; BQR—Brequinar; CMV—cytomegalovirus; CsA—Cyclosporin A;
CTL—cytotoxic T lymphocyte; DTH—delayed type hypersensitivity; ICAM—intercellular adhesion molecule;
IL—interleukin; LFA—lymphocyte function-associated antigen; mAb—monoclonal antibody;
MHC—major histocompatibility complex; MPA—mycophenolic acid; MZB—mizoribine;
NF-AT—nuclear factor of activated T cells; Pred—Prednisone; RAPA—Rapamycin; TCR—T-cell receptor; Th—T-helper.

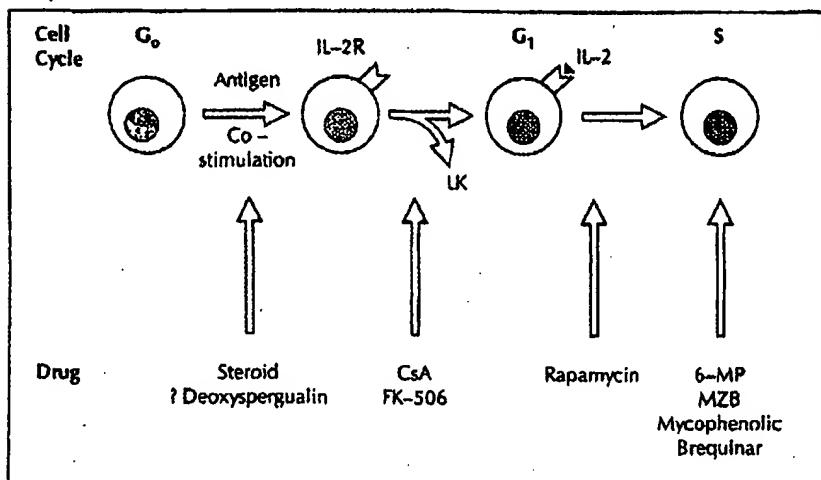


Fig. 1. Classification of immunosuppressive drugs based upon their site of action in the cell cycle. In the first group, corticosteroid, and possibly deoxyspergualin, inhibit antigen-presenting cells. In the second group, Cyclosporin A (CsA) and FK-506 inhibit lymphokine (LK) biosynthesis during the G₀ phase and Rapamycin inhibits signal transduction during the G₁ phase. In the third group (the nucleoside synthesis inhibitors), Mizoribine (MZB) and RS61443 (a morpholinoethyl-ether analog of mycophenolic acid, MPA) inhibit purine synthesis pathways leading to the generation of guanosine monophosphate, whereas Brequinar (quinoline carboxylic acid) inhibits the de novo synthesis of pyrimidines. IL, interleukin; R, receptor.

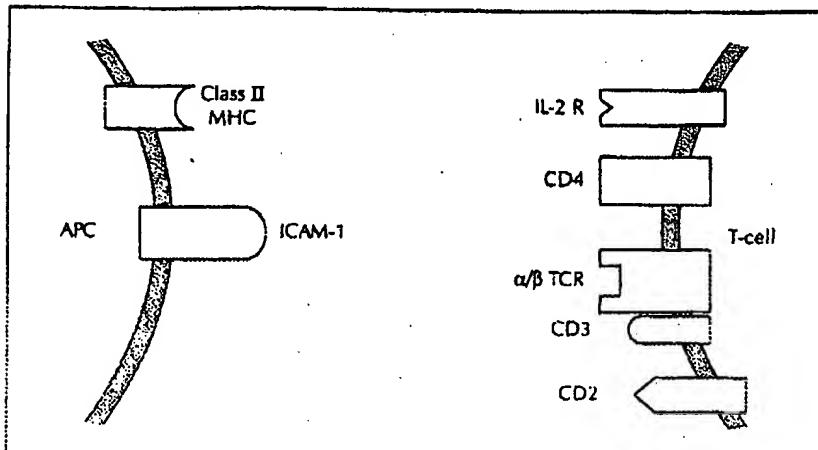


Fig. 2. Epitope targets of monoclonal antibodies. ICAM, intercellular adhesion molecule; IL, interleukin; MHC, major histocompatibility complex; R, receptor; TCR, T-cell receptor.

Table 1. Determinants recognized by immunosuppressive monoclonal antibodies

Monoclonal antibody	Determinant	Reference
IgM T1089.1.A-31	Determinants common to α/β chains of all human T-cell receptors	[4]
IgG2b BMA 031		[5]
OKT4A	CD4	[6]
SDZ CHH 380	CD7	[7]
33B3.1	α chain or α/β complex of interleukin-2 receptor	[8]
B1R-R-1	Intercellular adhesion molecule-1 (or CD54)	[9]
2S.3	Lymphocyte function-associated antigen-1	

equine or rabbit polyclonal anti-lymphocyte sera (ALG) and/or mouse OKT-3 mAbs have been combined in empirical regimens that are often tailored to individual patients. To address one limitation of CsA, nephro- and/or hepatotoxicity during the initial post-transplant phase of induction immunosuppressive therapy, which delays allograft and patient recovery, pharmacokinetic control

programs have been used either to pre-select CsA doses [11] or to combine ALG or OKT-3 with Aza/Pred in order to delay treatment with CsA. However, immediate, rather than delayed, administration of CsA to patients displaying good initial renal function avoids the disadvantages of ALG/OKT-3 induction, namely an increased risk of cytomegalovirus (CMV) infection, additional expense and/or delayed hospital discharge awaiting satisfactory CsA levels after treatment with CsA. However, patients at extraordinarily high immunological risk, due to rejection of previous allografts within three months, or with marginally-functioning organs may preferably be treated with ALG or OKT-3 induction as a possible means to delay the onset of their first rejection episodes. Two possible alternatives for induction therapy are the CsA analogs that may display reduced nephrotoxicity, namely Cyclosporin G [12], which substitutes norvaline at position 2, and IMM-125 with a hydrophilic substituent at position 9.

To date, no large randomized study has shown that the induction regimen alters the clinical outcome; rather, a small cohort reported by Belitsky *et al.* [13] showed no difference between ALG versus initial CSA therapy, the two options for induction therapy. There may be several reasons for this. Firstly, the polyclonal reagents, such as

rabbit or equine Minnesota ALG and anti-thymocyte globulin, regardless of their source, opsonize T cells, leading to their removal from the circulation. This depletion obviates T-cell mediated attack on the allograft. Among other factors that must be considered is that the central intravenous lines required for polyclonal administration may be accidentally contaminated, producing septicemia. Further, there are no indices of the efficacy of the polyclonal sera. Peripheral blood T-cell numbers above the target range of 50–150/ml tend only to be useful reflections of the appearance of human host anti-equine antibodies, not immunological resistance to therapy. The other induction therapy using OKT-3, which covers and/or modulates CD3 epitopes on the T-cell surface, offers the advantages of both peripheral intravenous administration and readily available fluorescence-activated cell sorting (FACS) tests for antibody efficacy. The clinician monitors patient peripheral blood lymphocyte T-cell epitopes for (a) cells with exposed CD3 epitopes that were not bound by OKT-3 *in vivo* by their capacity to bind fluorescinated OKT-3 *in vitro*, (b) the total number of circulating T cells with fluorescinated anti-CD2, a pan T-cell marker, and (c) the proportion of OKT-3-coated cells detected with a goat anti-mouse IgG reagent. A satisfactory therapeutic effect is observed when the patient has <25% OKT-3⁺ cells *in vitro* and 60–75% CD2⁺ T cells, about 40% of which are coated with mouse IgG which binds to OKT-3. While OKT-3 represents an advance in immunosuppressive therapy, it has several serious limitations: (a) severe first-dose reactions, including chills, fever, myalgias and, in the worst cases, pulmonary oedema apparently due to lymphokine release (particularly tumor necrosis factor and interleukin (IL)-2); (b) longer-term adverse effects such as aseptic meningitis; (c) a delay in the therapeutic effect for as long as 7 days after initiation of treatment; (d) induction of human anti-mouse antibodies, generally of the anti-idiotypic variety, but not uncommonly of broader reactivity; (e) a frequent incidence of rebound re-rejection episodes upon completion of the therapeutic course; and (f) a tendency toward CMV infections in 40% of treated patients. In addition, both polyclonal and mAb reagents may produce excessive immunosuppression, resulting in increased incidences of CMV infection and/or of lymphomas and other neoplasms, as well as allograft thrombosis. Thus, selection of a CsA versus an antibody induction regimen must balance the risks of nephrotoxicity versus that of excessive immunosuppression.

The use of CsA has reduced the risk of acute rejection, but a rational approach to CsA administration is confused by the tremendous variability between individuals in drug pharmacokinetics and pharmacodynamics [14], which, in turn, generates a fear of irreversible renal injury in case the CsA dose is excessive. Three approaches have been used to address this problem: (a) combining reduced CsA doses with subtherapeutic amounts of Aza [15]; (b) monitoring the parent compound CsA based upon its trough concentration prior to the next drug dose [16]; and (c) adjusting CsA doses prospectively based upon average concentrations calculated from serial measurements of the area under the concentration-time curve

[11]. Since drug absorption presents the greatest variability in pharmacokinetics, attempts have been made to increase CsA bioavailability by co-administration of Vitamin E [17]. In addition, the manufacturer has produced a new micro-emulsion formulation that increases the bioavailability by twofold above that of the existing oral solution or capsule preparation, which show equivalent bioavailability in studies comparing both formulations [18]. The critical issue seems to be the drug concentration in the allograft. While direct intra-arterial infusion has been used for experimental models of renal or cardiac allografts, good drug uptake in man can be achieved by presenting CsA as an aerosol in absolute ethanol when it has a mean particle diameter of 1.2 microns [19].

Optimal use of CsA demands the measurement of drug concentrations/activities at the level of its lymphocyte receptor or target signal transduction molecule(s), which may be calcineurin (an enzyme that may be involved in a common step associated with T-cell and IgE receptor signalling pathways) [20•] or the nuclear factor of activated T-cells (NF-AT). However, a major limitation may be the failure of CsA to inhibit lymphocyte activation via the CD28 surface marker [21], an important co-stimulatory pathway that together with T-cell receptor (TCR) stimulation blocks induction of anergy in T-cell clones [22]. Fortunately, rejection episodes under CsA prophylactic therapy tend to be readily reversed by corticosteroid therapy, and the majority of steroid-resistant episodes are overcome with polyclonal ALG and/or OKT-3 therapy. Corticosteroids are believed to represent the Achille's heel of transplantation because of the wide distribution and pleiotropic effects of the glucocorticoid receptor superfamily found in the cytoplasm. These are DNA-binding dimeric transcription factors with a zinc finger structure that recognize enhancer (or negative regulator) elements bearing the GRE motif (GTACAnnnT-GTTCT, where n = any nucleotide). One important negative regulatory element is the AP-1 binding site, normally the focus for fos-jun heterodimers [23]. An alternative approach to the reduction of IL-1 β generation, an action typical of corticosteroids, is to inhibit the enzyme that cleaves the inactive 31 kD precursor between Asp¹¹⁶ and Ala¹¹⁷ to release the 153 carboxyl-terminal amino acids that constitute IL-1 β [24]. Another immunosuppressive effect may be achieved by the upregulation of the synthesis of transforming growth factor- β by steroids [25]. Withdrawal of steroid treatment months to years after the transplant may be successful in patients who did not reject the transplant [26•].

Preliminary data suggest that a ten-day course of the IgM mouse anti-human α/β TCR mAb T10B9.1A-31 [4], but not BMA031 (C Groth, personal communication), not only produces equivalent therapeutic effects to those of OKT-3, but is less toxic in terms of incidence of fever and neurological and respiratory symptoms, as well as of subsequent infections. Furthermore, T10B9 therapy is not associated with as great a rise in serum creatinine during treatment as OKT-3, suggesting a more rapid attenuation of the allo-immune response. However, the repeated use of xenogeneic antibodies during the induc-

tion phase as well as for anti-rejection therapy may be complicated by the development of neutralizing human anti-mouse antibodies.

A major goal of maintenance immunosuppressive therapy is prophylaxis against chronic rejection. To date, not only has CsA/Pred therapy failed to reduce the incidence of this complication from the 8–10% level observed under the Aza/Pred combination, but there is no way to determine if the failure is due to its inherently modest inhibition of B-cell responses or to physicians' tendency to limit CsA therapy to minimal, possibly ineffective, doses in order to mitigate a renal injury. Thus, despite the improvement in initial graft survival, transplants continue to be lost in the longer term, with half-lives of about seven years for cardiac and 11.5 years for renal transplant in humans. A recent study of the effects of immunosuppressive drugs on coronary vascular disease in heterotopic rat cardiac allografts suggests that RAPA particularly, CsA to a lesser extent, but definitely not FK-506, inhibit pathological endothelial and smooth muscle lesions in arteries and arterioles, which seem to be the critical lesions in the progression of chronic rejection [27].

New pharmacological agents

Both the macrolide FK-506 and the undecapeptide CsA interrupt lymphokine synthesis by inhibiting generation of the Ca^{2+} dependent regulatory proteins NF-AT, NFIL-2A, NFIL-2B, and NF- $\kappa\beta$, but not c-fos, which is necessary for IL-2 generation. Presumably, both drugs also affect serine protease gene transcripts, an excellent marker of rejection [28]. The inhibition of cytotoxic T lymphocytes (CTLs), even in the presence of optimal amounts of IL-2, is a prominent effect of CsA [29] and, apparently, FK-506. Despite the assumption that CsA and FK-506 produce similar inhibitory effects, at least three differences have been observed: first, FK-506 displays a flatter inhibition curve than CsA with a wider discrepancy in potency at the 50% inhibition than at the 95% inhibition level; secondly, CsA leads to the generation of suppressive T cells, whereas FK-506 does not; and thirdly, although both drugs inhibit $\text{CD}4^+$ T helper (Th) lymphocytes, which secrete IL-2, only CsA (and not FK-506) permits priming of $\text{CD}8^+$ CTLs [30]. Furthermore, Bretscher and Havele [31] suggest that CsA switches the immune response to the graft from a delayed type hypersensitivity (DTH) response to an IgG response by inhibiting the Th1 subset with the emergence of the Th2 subset, which actively induces IgG via IL-4 generation and inhibits Th1 cells and DTH via IL-10. Both CsA and FK-506 spare transcription of the down-regulatory lymphokine IL-10. While CsA inhibits transcription of IL-6, this factor is not affected by FK-506.

The coming year should witness publication of a vast array of randomized trials comparing the clinical outcome of liver and renal transplants in patients treated with FK-506 versus CsA. So far, a preliminary non-randomized study of liver recipients showed that FK-506 therapy displays greater neurotoxicity, equivalent nephrotoxicity,

but, possibly, less hypertension than does CsA therapy [32*]. A further claim that corticosteroids do not have to be used with FK-506 cannot be assessed due to two factors: firstly, the protocol stipulated higher Pred doses in the CsA cohort than those used with FK-506; and secondly, to date, there is no pharmacokinetic analysis of Pred concentrations in CsA versus FK-506 treatment groups in order to exclude a drug interaction. Additionally, the extremely poor results in the initial study, wherein allegedly CsA-resistant patients were converted to treatment with FK-506, actually reflected antagonism between the two drugs caused by (a) an adverse immunological interaction between the two agents that apparently have similar mechanisms of action [33] and (b) competitive pharmacokinetic interactions. Although FK-506 has not yet been shown to achieve clinical results even equivalent to those of CsA, eventual definition of its relative therapeutic window will depend upon Phase II studies to select well-tolerated drug doses for randomized trials versus CsA therapy.

When a second agent, RS61443, was added in doses of 2 500–3 500 mg per day to a CsA/Pred regimen, it seemed to reduce the incidence of acute rejection episodes. However, these high doses are likely to produce toxicity, particularly leukopenia and gastrointestinal complaints [2*]. Randomized placebo-controlled trials are underway to assess the efficacy of RS61443 versus Aza added to a CsA/Pred regimen. Other studies are examining the impact of a fourth agent, deoxyspergualin, to potentiate an ALG/Aza/Pred/CsA induction protocol.

The studies that claimed Aza displays pharmacological synergism with CsA failed to utilize rigorous experimental design or data analysis [34]. For instance, both *in vitro* analyses [35] and clinical results demonstrate that Aza acts in an additive manner rather than synergistically with CsA [36]. Similarly, *in vitro* analyses suggest that RS61443 [37], mizoribine [37], and thalidomide [38] also act in an additive manner with CsA. Although initial data suggested that BQR potentiates the effect of CsA [30*], recent experiments document true synergism [37]. However, CsA/RAPA combinations show the most impressive degree of synergy both *in vitro* and *in vivo* [39]. Once Phase I toxicity trials have been completed, it will be possible to assess whether BQR or RAPA displays the synergistic effects with CsA in human transplantation that are evident in rodents and large animal models.

New monoclonal antibody reagents

Second generation mAbs are being designed to avoid the severe systemic reactions due to lymphokine release that follow initial doses of OKT-3. For example, the IgG2b anti-human α/β TCR mAb BMA 031 used for induction therapy (three 50 mg doses administered on alternate days) delays the onset of first rejection episodes and probably improves one-year graft survival (R Knight and BD Kahan, unpublished data). Similar benefits have been reported with mouse and rat mAbs produced against the activation-induced α -chain, or to new epitopes resulting

from the formation of the $\alpha\beta$ complex, of the IL-2 receptor [8].

However, treatment with these antibodies leads to a high incidence of human anti-mouse antibodies, which may attenuate the immunosuppressive effects. Recent work has explored approaches to construct either (a) 'chimeric' antibodies bearing human Fc segments joined to mouse F(ab')₂ fragments, or (b) 'humanized' mAbs with mouse idiotypes inserted onto human IgG isotypes (Fig.3). Chimeric antibodies combine the variable regions of mouse antibodies with human antibody constant regions and, therefore, present fewer foreign amino acid sequences to the host. However, one-third of the structure is still of mouse origin. Furthermore, a clinical trial using a chimeric anti-CD7 mAb not only failed to achieve a superior level of immunosuppression induction, but also increased the incidence of vascular thromboses [7]. The latter effect may have been related to the adhesion of Fc receptors on platelets and polymorphonuclear leukocytes to the human Fc regions, bound to endothelium via mouse epitopes. On the other hand, 'humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen combining site, with human variable region frameworks and constant regions. Due to the reduced affinity of 'humanized' antibodies for antigen epitopes, Co *et al* [40] recommended two innovations: firstly, selection of a human framework that is as homologous to the original mouse antibody as possible; and secondly, insertion of key residues from the mouse model into the construct in order to achieve a molecular conformation that is similar to the native idiotype. The beneficial effects of chimeric and 'humanized' variants of mouse mAbs will be clarified only by randomized clinical trials.

Two alternative approaches seek to utilize mAbs directed against donor MHC antigens or against co-receptor molecules. In a study of non-human primates,

OKT-4A IgG2A mAbs, which react with the CD4 co-receptor on Th cells, provoked fewer side effects than OKT-3 [6]. In an initial clinical trial of OKT-4A induction therapy (0.2 mg/kg/day), all six patients suffered rejections. These rejections were reversible, but left residual areas of dead tissue resulting from an obstruction of the blood supply in half the renal allografts. Unfortunately, OKT-4A also generated a strong human anti-mouse antibody response (J Barry, personal communication). Experimental animal models are currently being used to determine if antibody efficacy is related to T-cell deletion and is potentiated by simultaneous treatment with an anti-CD8 mAb. On the one hand, Fathman and colleagues [41] found that depleting anti-CD4 mAbs produced prolonged allo-unresponsiveness toward allogeneic pancreatic islets, an effect that was moderated by simultaneous treatment with anti-CD8⁺ mAbs, suggesting the role of a regulator CD8⁺ cell. On the other hand, Waldmann and colleagues [42] induced tolerance toward mouse heart transplants where the donor and recipient were not matched at MHC level using an anti-CD4 mAb that not only did not deplete T cells but also was potentiated by simultaneous administration of an anti-CD8 mAb.

A second approach to co-receptor molecules is based on the interaction of lymphocyte function-associated antigen (LFA)-1 on CTLs with the intercellular adhesion molecule (ICAM)-1 or monocytes. Expression of ICAM-1 is up-regulated following lymphokine release, which occurs during acute allograft rejection but not during other pathological events in the kidney [43**]. Prophylactic and therapeutic administrations of a mAb directed against the high molecular weight α -chain of human ICAM-1 alone delayed both the onset and progression of rejection episodes in primate renal allograft models. Using mouse mAbs directed against LFA-1, Stoppa *et al* [44] reversed steroid resistant acute graft versus host reactions in man. Indeed, the combination of anti-ICAM-1 and anti-LFA-1 mAbs produced allo-tolerance in mice that were not com-

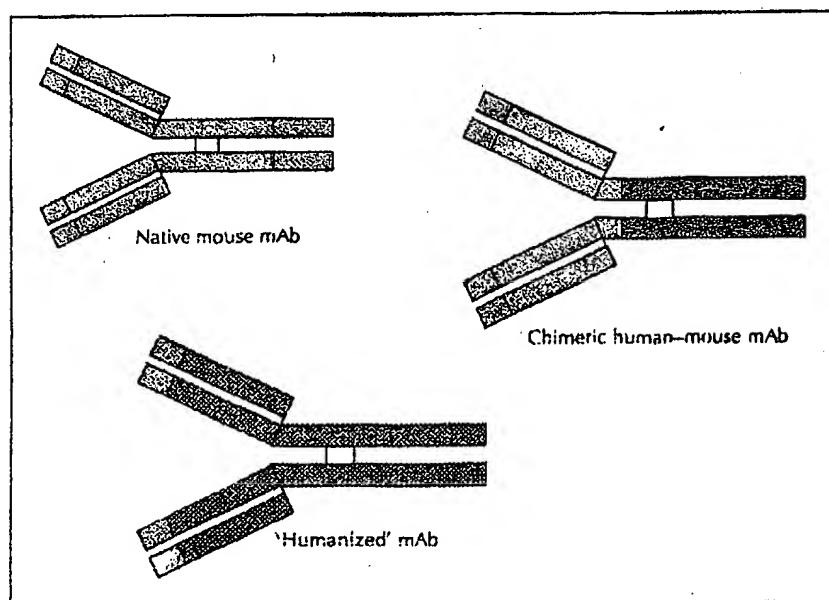


Fig. 3. Types of monoclonal antibody. Chimeric antibodies combine the variable regions of mouse antibodies with human constant regions and, therefore, present fewer foreign amino acid sequences to the host. 'Humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen binding site, with human variable region frameworks and constant regions.

patible at the MHC level [45••]. These promising results in animal models using mAbs directed against T-cell and monocyte co-receptors await confirmation in controlled clinical trials.

While clinical interventions to date have focused on using mAbs directed towards surface epitopes important for the afferent limb of the allo-immune response, there is increasing evidence that anti-idiotypic antibodies, either exogenously introduced or endogenously, spontaneously generated, may regulate the induction of allo-immune responses. A recent study performed by Snider [46] suggested that immunization of hosts with antigen-antibody complexes confers a bias in the epitope, resulting in a less efficient antibody response that shows anti-idiotypic properties. This approach represents a particularly fertile ground for clinical exploration.

Cytokine receptor analogs and antagonists

A new group of immunosuppressive agents are the cytokine receptor antagonists. The discovery and initial testing of an IL-1 receptor antagonist has been reviewed by Arend [47]. IgG-stimulated human monocytes naturally produce IL-1 receptor antagonist, a heterogenous array of glycoproteins of 15–25 kD, depending upon their degree of glycosylation. IL-1 receptor antagonist binds type I, but not type II, IL-1 receptors without activating cells and with considerably less avidity than native IL-1 α and IL-1. Type I IL-1 receptors are present on Th2 cells and fibroblasts; Type II IL-1 receptors are present on B cells, neutrophils, and macrophages. Although therapeutic trials of IL-1 receptor antagonist in rheumatoid arthritis and septic shock suggest some beneficial effects, Faherty *et al.* [48] failed to observe that IL-1 receptor antagonist inhibited induction of CTLs, cutaneous DTH, or T-cell dependent humoral antibody responses. They also found that administration of a mAb to type II IL-1 receptor (35F5) was ineffective. Fanslow *et al.* [49•] recently extended their previous studies, which used constructs of the extra-membranous portion of the IL-1 receptor, by using similar constructs of the IL-4 receptor. In the initial studies, they prolonged heterotopic pinnal, neonatal mouse heart allograft survival, but failed to prevent allo-sensitization, as documented by a rapid, secondary-type proliferative response upon *in vitro* one-way mixed lymphocyte reactions. In their recent studies, constructs of IL-4 receptor alone, or in combination with rat anti-mouse IL-4 receptor mAb, induced modest prolongation of heart allo-explants.

Immunosuppressive drug trials

Of the numerous obstacles currently hindering the development of efficacious immunosuppressive regimens, the lack of methodology for clinical transplantation trials is of particular importance. To date, no series of Phase I and II toxicity and dose-finding trials has been conducted in

order to establish a foundation for clinical investigation. The introduction of Aza, steroids, and CsA, as well as the preliminary trials of FK-506, have relied upon empirical approaches. Important obstacles to comprehensive trials include the relatively small numbers of transplant cases, the use of unrefined end-points such as graft and patient survival, and the lack of well-established criteria for the diagnosis and grading of rejection episodes, deficits that obfuscate the use of this event as an intermediate endpoint. In addition, no *in vitro* immune assay predicts or correlates with *in vivo* immunosuppressive efficacy; hence, there is no surrogate immune parameter as a basis of immunosuppressive efficacy and/or for dose extrapolation from *in vitro* systems to *in vivo* conditions.

Since present results with CsA-based regimens yield excellent graft survivals, extremely large numbers of patients must be entered into clinical trials to document improved efficacy of a new agent. Even more extensive efforts will be needed to exclude the possibility that the results with the new agent are not actually worse than those obtained with the existent CsA regimen. In light of the presently high success rates, the benefits of any new regimen must be based upon both the potency and the mitigation of side effects, as assessed by quantitative parameters, including glomerular filtration rates. The practice of clinical research in transplantation must proceed to develop principles of rigorous study design and precise analytical tools in order to most expeditiously evaluate the available array of new immunosuppressants described in this review.

Acknowledgements

This study was supported by an award from the National Institute of Diabetes, Digestive and Kidney Diseases NIDDK38016-05. The author acknowledges the invaluable editorial assistance of Ms. B. Lee Ligon.

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BD Kahan, Department of Surgery, Division of Immunology and Organ Transplantation, University of Texas Health Science Center at Houston, Houston, Texas 77030, USA.

INTERLEUKIN-12 (IL-12)-DRIVEN ALLOIMMUNE RESPONSES IN VITRO AND IN VIVO

REQUIREMENT FOR $\beta 1$ SUBUNIT OF THE IL-12 RECEPTOR¹

JOSEPH R. PICCOTTI,^{2,3} KEWANG LI,² SHERRI Y. CHAN,² ERNST J. EICHWALD,⁴ AND D. KEITH BISHOP^{2,5}

Department of Surgery, Section of General Surgery and Department of Microbiology and Immunology, University of Michigan School of Medicine, Ann Arbor, Michigan 48109, and Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84132

Background. Interleukin-12 (IL-12) mediates its biologic activities via binding high-affinity receptors on T and natural killer cells. Although emphasis has been placed on the requirement for IL-12R $\beta 2$ in IL-12 bioactivity, the role of IL-12R $\beta 1$ is less well defined. The current study evaluated the effects of exogenous IL-12 on alloantigen-specific immune responses and determined the requirement for IL-12R $\beta 1$ in IL-12-mediated alloimmunity.

Methods. The mouse heterotopic cardiac transplant model was employed to evaluate the effects of IL-12 on alloantigen-specific immune responses *in vivo*. In addition, IFN- γ production in mixed lymphocyte cultures (MLC) supplemented with IL-12 was measured to assess the effects of IL-12 on Th1 function *in vitro*. Mice deficient in IL-12R $\beta 1$ (IL-12R $\beta 1^{-/-}$) were used to determine the requirement for this receptor component in IL-12-driven alloimmune responses.

Results. Addition of IL-12 to MLC consisting of wild-type splenocytes enhanced alloantigen-specific proliferative responses and Th1 development. In contrast, IL-12 did not alter these *in vitro* immune parameters in IL-12R $\beta 1^{-/-}$ MLC. Treatment of wild-type cardiac allograft recipients with IL-12 resulted in high concentrations of serum interferon- γ (IFN- γ) and a 10-fold increase in IFN- γ production by recipient splenocytes after restimulation *in vitro*. However, this fulminate Th1 response did not accelerate allograft rejection. Importantly, IL-12 had no effect on serum IFN- γ or *in vivo* priming of Th1 in IL-12R $\beta 1^{-/-}$ recipients. Finally, administration of IL-12 to WT allograft recipients resulted in a bimodal alloantibody response: antibody production was suppressed at high doses of IL-12, and enhanced at lower doses.

Conclusions. IL-12 markedly enhances alloantigen-specific immune function; however, these exaggerated Th1-driven responses do not culminate in accelerated allograft rejection. Further, these data indicate that IL-12R $\beta 1$ is essential for the enhancement of both *in*

¹ This work was supported by grant R01 AI31946 (D.K.B.) from the National Institutes of Health.

² Department of Surgery, Section of General Surgery, University of Michigan School of Medicine.

³ Address correspondence and reprint requests to Joseph R. Piccotti, Ph.D., Transplant Immunology Research Laboratory, Section of General Surgery, A560 MSRB II, Box 0654, University of Michigan Medical Center, Ann Arbor, MI 48109-0654.

⁴ Department of Pathology, University of Utah School of Medicine.

⁵ Department of Microbiology and Immunology, University of Michigan School of Medicine.

vitro and *in vivo* alloimmune responses by exogenous IL-12.

It is well established that interleukin-12 (IL-12*) is a critical cytokine involved in the regulation of Th1- and Th2-mediated immune responses in several experimental models (reviewed in 1 and 2). IL-12 has direct stimulatory and inhibitory effects on Th1 and Th2, respectively (3-6). Further, this cytokine promotes Th1 and inhibits Th2 development indirectly by inducing interferon- γ (IFN- γ) production by activated T cells and natural killer cells (7-12). Th1 have been accepted as key regulators of allograft rejection, in that this cell type promotes both delayed-type hypersensitivity and cytotoxic T lymphocyte responses, which are believed to be the principle terminal effector mechanisms of acute allograft rejection (13, 14). An understanding of the role of IL-12 in graft rejection is just emerging. For example, IL-12 clearly augments alloreactive Th1 development *in vitro* (15). However, the presence of IL-12 is not mandatory for the development of acute cardiac allograft rejection (15, 16). Hence, an important question is whether enhanced Th1 function alters the rejection response. Given the IL-12/Th1 dogma, one would predict that IL-12 would augment alloreactive Th1 function, resulting in accelerated allograft rejection. The present study therefore was designed to test the hypothesis that IL-12-driven Th1 responses would exacerbate cardiac allograft rejection.

IL-12 mediates its biologic effects by interacting with a high-affinity receptor, which consists of at least two cloned components, IL-12R $\beta 1$ and IL-12R $\beta 2$ (17-19). IL-12R $\beta 1$ interacts with the p40 subunit of IL-12, whereas the p35 subunit of IL-12 is believed to bind to IL-12R $\beta 2$ (19, 20). Emphasis has been placed on the necessity for IL-12R $\beta 2$ in IL-12 signaling (21, 22). However, by utilizing IL-12R $\beta 1$ knockout mice (IL-12R $\beta 1^{-/-}$), Wu et al. (23) recently reported that the $\beta 1$ subunit of IL-12R is essential for IL-12-driven proliferation and IFN- γ production by mitogen-activated blasts, natural killer cell lytic activity, and IFN- γ production in response to endotoxin. We have reported that the p40 subunit of IL-12 stimulates alloreactive CD8+ Th1 development both *in vitro* (24) and *in vivo* (15). These observations suggest that

* Abbreviations: Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; GVHD, graft-versus-host disease; H&E, hematoxylin and eosin; IFN- γ , interferon- γ ; IL, interleukin IL-12R $\beta 1^{-/-}$, mice deficient in $\beta 1$ subunit of IL-12 receptor; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; WT, wild-type.

IL-12R β 1 may be needed for alloreactive Th1 development, and that signaling through IL-12R β 1 may be sufficient to mediate IL-12's biologic activity on CD8+ T cells. Hence, the present study employed IL-12R β 1 $^{-/-}$ mice to determine whether β 1 subunit of IL-12R is required for IL-12-induced alloantigen-specific immune responses. To our knowledge, this study is the first to investigate the effects of IL-12 treatment on alloreactive Th1 development *in vivo* and to establish a mandatory role for IL-12R β 1 in IL-12-driven alloimmune responses.

MATERIALS AND METHODS

Mice. Wild-type (WT) C57BL/6 and BALB/c mice between 6 and 12 weeks of age were obtained from Charles River Laboratories (Raleigh, NC). Generation of C57BL/6 IL-12R β 1 $^{-/-}$ mice has been described previously (23). These mice were generated on the 129/Sv background and back-crossed to C57BL/6 mice for five generations, then intercrossed to generate homozygotes.

Medium. The culture medium used in these studies was Dulbecco's minimum essential medium supplemented with 1.6 mM L-glutamine, 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 μ M folic acid, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, 2% fetal calf serum (all obtained from Life Technologies, Grand Island, NY), and 5 \times 10 $^{-5}$ M 2-mercaptoethanol (Sigma Chemical, St Louis, MO).

Mitogen-driven cytokine production. To investigate the requirement for the β 1 subunit of IL-12R in mitogen-stimulated IL-10 and IFN- γ production, splenocytes (2×10^6 cells/ml) isolated from naive WT or IL-12R β 1 $^{-/-}$ C57BL/6 mice were incubated for 72 hr with 1 μ g/ml concanavalin A (Con A) (Sigma Chemical). Cultures were supplemented with 1 ng/ml murine recombinant IL-12 (rIL-12) (kindly provided by Dr. Maurice Gately, Hoffmann-La Roche Inc.) to assess the effect of exogenous IL-12 on Con A-stimulated cytokine production by splenocytes of WT and IL-12R β 1-deficient mice. Resulting supernatants were harvested at 72 hr, and the concentrations of IL-10 and IFN- γ measured by enzyme-linked immunosorbent assay (ELISA).

In vitro alloimmune responses. To assess alloantigen-specific Th1 development, splenocytes (1×10^6 cells/ml) isolated from naive WT or IL-12R β 1 $^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated (5000 rads) BALB/c splenocytes (1×10^6 cells/ml). Where indicated, 1 ng/ml of murine rIL-12 was added to primary mixed lymphocyte cultures (MLC) to assess the effect of exogenous IL-12 on alloantigen-driven Th1 function and to evaluate whether Th1 from IL-12R β 1-deficient mice were responsive to IL-12 stimulation. The concentration of rIL-12 was selected from dose-response experiments in which the amount of rIL-12 needed for maximal enhancement of alloantigen-specific proliferation was 5–10 ng/ml (data not shown). Resulting cell populations were harvested, washed three times, and restimulated (at 1×10^6 cells/ml) with irradiated BALB/c stimulator cells (1×10^6 cells/ml). MLC supernatants were collected after 24 hr (IL-4 and IL-10) or 72 hr (IFN- γ), and cytokine concentrations measured by ELISA.

In addition, splenocyte proliferative response to alloantigens was determined in cultures either left unmodified or supplemented with 1 ng/ml murine rIL-12. WT or IL-12R β 1 $^{-/-}$ C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated for 5 days with irradiated BALB/c splenocytes (1×10^6 cells/ml) in 96-well U-bottom plates (Becton Dickinson, Lincoln Park, NJ) in a final volume of 200 μ l (done in quadruplicate). Cultures were pulsed with 0.5 μ Ci/well [methyl^3H]thymidine (ICN, Costa Mesa, CA) for the final 8 hr of the incubation period. [methyl^3H]Thymidine incorporation was assessed on a Wallac 1205 Betaplate scintillation counter (Wallac, Turku, Finland).

Heterotopic cardiac transplantation. Intact BALB/c (H2 d) hearts were anastomosed to the great vessels in the abdomens of WT or

IL-12R β 1 $^{-/-}$ C57BL/6 (H2 b) mice as described by Corry et al. (25). In this model, the transplanted heart is perfused with the recipient's blood and resumes contractions until acutely rejected, which occurs in unmodified WT recipients of this strain combination in approximately 8–9 days (15, 24). Graft function was evaluated by daily abdominal palpation. Myocyte damage and intensity of graft-infiltrating cells were assessed by routine hematoxylin and eosin (H&E) staining of paraffin-embedded sections of transplanted allografts.

Experimental groups. Cardiac allograft recipients were divided into four groups: (1) recipients injected intraperitoneally with 1 mg of anti-CD8 monoclonal antibody (mAb) (hybridoma 2.43, purified by Montana ImmunoTech Inc., Bozeman, MT) on days -2 and -1 before transplantation, (2) animals given daily intraperitoneal injections of murine rIL-12 (0.1 or 1.0 μ g) on days 1–6 after transplantation, (3) recipients injected with a combination of 2.43 anti-CD8 mAb plus rIL-12, and (4) unmodified (no treatment) mice, which served as controls. Depletion of CD8+ cells (<2%) was verified by flow cytometry using anti-CD8:fluorescein isothiocyanate antibody (PharMingen).

In vivo alloimmune responses. To monitor in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from allograft recipients were restimulated with irradiated BALB/c stimulator cells (1×10^6 cells/ml), and the concentration of IFN- γ was measured by ELISA. As an additional measure of the in vivo activity of IL-12 on IFN- γ production, sera IFN- γ concentrations in WT and IL-12R β 1 $^{-/-}$ cardiac allograft recipients were measured by ELISA. Further, to assess the effect of IL-12 treatment on B cell function, sera alloantibody (IgM, IgG1, and IgG2a) levels were determined (see below).

Cytokine ELISA. Experimental samples (100 μ l) were added in triplicate to plates coated with 5 μ g/ml rat anti-mouse IFN- γ , IL-4, or IL-10 capture antibodies (PharMingen). Standards were employed by preparing 2-fold dilutions of murine recombinant IFN- γ , IL-4, and IL-10 (PharMingen), with a starting concentration of 25, 2.5, and 10 ng/ml, respectively. After a 1-hr incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS. One hundred microliters of rat anti-mouse secondary biotinylated antibodies (1 μ g/ml) (PharMingen) was then added, and plates were incubated at room temperature for 45 min. Plates were then washed three times with 0.05% Tween 20 in PBS, and 100 ml of avidin-peroxidase (Sigma Chemicals) was added. After a 30-min incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS, and 100 ml of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma Chemical) was then added to each well. After 20 min, absorbance was determined at 405 nm by an EL 800 microplate reader (Bio-Tek Instruments, Winooski, VT). Sample cytokine concentrations were calculated from a standard curve. The sensitivity of this assay is approximately 300 pg/ml for IFN- γ , 100 pg/ml for IL-4, and 150 pg/ml for IL-10.

Sera alloantibody determination. P815 cells (H2 d) were stained for flow cytometric analysis using dilutions of sera (1:50) obtained from cardiac allograft recipients as the primary antibody, followed by fluorescein isothiocyanate-conjugated isotype-specific anti-mouse IgM, IgG1, and IgG2a secondary antibodies (The Binding Site, San Diego, CA). Data are reported as the mean channel fluorescence determined on a Becton Dickinson FACScan.

Statistics. Statistical analyses in this study were done using a Student's *t* test performed by the program StatView 4.1.

RESULTS

Requirement for IL-12R β 1 in T Cell Responses *in Vitro*

Enhancement of mitogen-driven IFN- γ and IL-10 production by IL-12 requires IL-12R β 1. IL-12 stimulates concomitant production of IL-10 and IFN- γ by activated T cells (15, 26, 27). To determine whether β 1 subunit of IL-12R is required for production of these cytokines, C57BL/6 splenocytes isolated WT or IL-12R β 1-deficient mice were stimu-

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lated with Con A for 72 hr, and supernatant cytokine concentrations were determined by ELISA. Production of the Th1 cytokine IFN- γ by Con A-stimulated splenocytes isolated from IL-12R $\beta 1^{-/-}$ mice was readily detectable (Fig. 1A), although concentrations were lower than that seen in WT controls (IL-12R $\beta 1^{-/-}$ = 1.03 ng/ml vs. WT = 5.24 ng/ml). Addition of exogenous rIL-12 significantly enhanced IFN- γ production by mitogen-stimulated splenocytes obtained from WT mice (15.79 ng/ml). In contrast, IFN- γ production by splenocytes from IL-12R $\beta 1^{-/-}$ mice was not altered after the addition of rIL-12 (1.54 ng/ml).

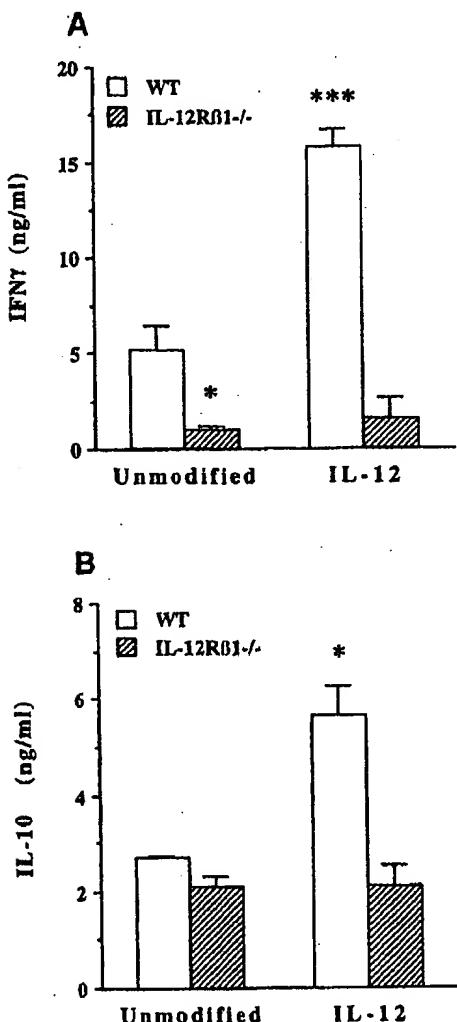


FIGURE 1. Mitogen-driven cytokine production by splenocytes isolated from IL-12R $\beta 1^{-/-}$ mice. Splenocytes (2×10^6 cells/ml) obtained from WT or IL-12R $\beta 1^{-/-}$ C57BL/6 mice were stimulated in vitro with 1 μ g/ml Con A. Cultures were either left untreated or supplemented with murine rIL-12 (1 ng/ml). Supernatants were collected after 72 hr, and the concentrations of IFN- γ (A) and IL-10 (B) were determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples \pm SD. Data are representative of three separate experiments. In panel A, *, P < 0.05 (WT unmodified vs. IL-12R $\beta 1^{-/-}$ unmodified); ***, P < 0.005 (WT unmodified vs. WT IL-12-treated). In panel B, *, P < 0.05 (WT unmodified vs. WT IL-12-treated).

The requirement for IL-12R $\beta 1$ in IL-12-driven IL-10 production also was assessed. Splenocytes isolated from IL-12R $\beta 1$ -deficient mice produced similar levels of IL-10 upon Con A stimulation when compared to WT cells (Fig. 1B). rIL-12 enhanced Con A-stimulated IL-10 production by WT splenocytes (2.70 ng/ml vs. 5.65 ng/ml). However, the $\beta 1$ subunit of IL-12R was required for this response, as IL-12 did not affect IL-10 secretion by mitogen-stimulated IL-12R $\beta 1^{-/-}$ splenocytes.

In vitro alloreactive T helper cell development. To evaluate the requirement for IL-12R $\beta 1$ in IL-12-driven alloantigen-specific T cell development, naive splenocytes obtained from WT or IL-12R $\beta 1^{-/-}$ mice were incubated for 5 days with irradiated BALB/c splenocytes in primary MLC, which were either left unmodified or supplemented with rIL-12. Resulting cell populations were restimulated with irradiated BALB/c splenocytes in the absence of rIL-12, and in vitro IFN- γ , IL-4, and IL-10 production determined by ELISA (Table 1). Primed WT splenocytes secreted high levels of IFN- γ upon restimulation with alloantigens. Splenocytes obtained from IL-12R $\beta 1^{-/-}$ mice secreted IFN- γ upon restimulation with alloantigens, albeit to a lesser degree than WT cells (WT = 21.32 ng/ml vs. IL-12R $\beta 1^{-/-}$ = 5.72 ng/ml). The decrease in alloantigen-stimulated IFN- γ production in IL-12R $\beta 1$ -deficient mice was not associated with a decrease in the cells' ability to proliferate in response to alloantigens (Fig. 2), in that [³H]thymidine incorporation by alloantigen-stimulated IL-12R $\beta 1^{-/-}$ splenocytes was similar to that seen by WT cells (IL-12R $\beta 1^{-/-}$ = 13,385 cpm vs. WT = 11,441 cpm). In both groups, IL-4, IL-10 (Table 1), and IL-5 (data not shown) were not detected in cultures that were not supplemented with exogenous rIL-12.

As shown in Table 1, exogenous rIL-12 markedly enhanced IFN- γ production by WT splenocytes in vitro (21.32 ng/ml vs. 215.13 ng/ml), but failed to augment IFN- γ secretion by cells obtained from IL-12R $\beta 1^{-/-}$ mice (5.72 ng/ml vs. 6.80 ng/ml). Likewise, rIL-12 significantly enhanced WT splenocyte proliferation in the MLC (Fig. 2), but did not alter the proliferative ability of splenocytes isolated from IL-12R $\beta 1$ -deficient mice. Finally, the addition of exogenous rIL-12 to cultures

TABLE 1. IL-12 does not enhance alloantigen-specific Th1 development in IL-12R $\beta 1^{-/-}$ mice in vitro^a

Treatment	IFN- γ (ng/ml)	IL-4 (ng/ml)	IL-10 (ng/ml)
Unmodified			
WT	21.32 \pm 0.88	ND	ND
IL-12R $\beta 1^{-/-}$	5.72 \pm 0.24**	ND	ND
IL-12 (1 ng/ml)			
WT	215.13 \pm 13.52***	ND	1.54 \pm 0.21
IL-12R $\beta 1^{-/-}$	6.80 \pm 0.21	ND	ND

^a Splenocytes (1×10^6 cells/ml) obtained from WT or IL-12R $\beta 1^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated allogeneic splenocytes (1×10^6 cells/ml) in unmodified MLC or MLC supplemented with murine rIL-12 (1 ng/ml). Resulting cell populations were harvested and restimulated with alloantigens for cytokine determination. Supernatants concentrations of IFN- γ (72 hr), IL-4 (24 hr), and IL-10 (24 hr) were measured by ELISA. Results are expressed as the cytokine concentration in triplicate samples (mean \pm SD). Data are representative of four separate experiments. ND indicates not detectable. **, P < 0.01, WT unmodified vs. IL-12R $\beta 1^{-/-}$ unmodified; ***, P < 0.005, WT unmodified vs. WT IL-12-treated.

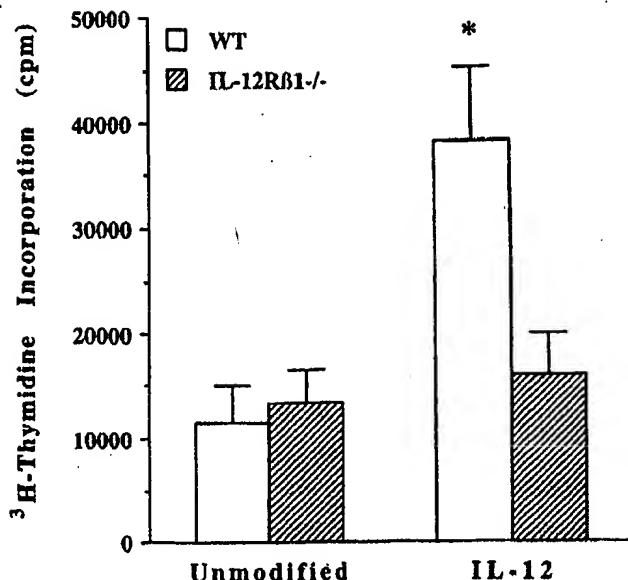


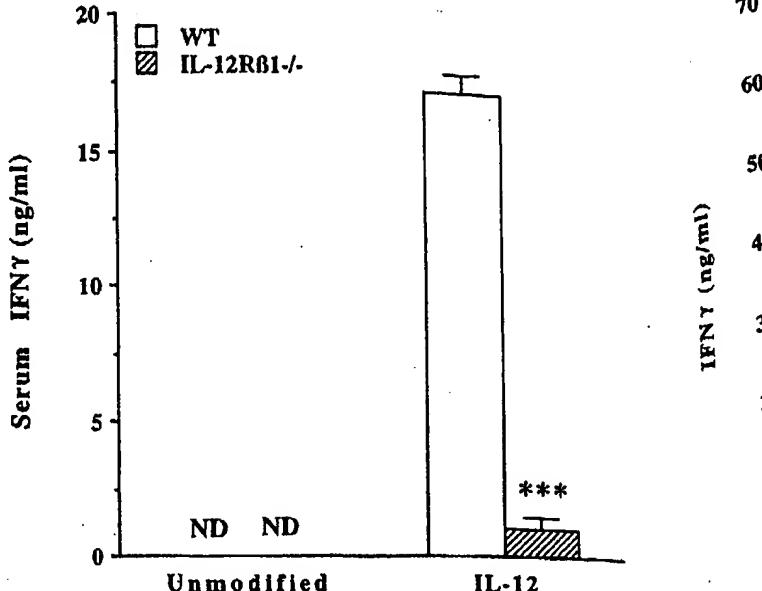
FIGURE 2. The β 1 subunit of IL-12 receptor is required for IL-12-induced stimulation of alloantigen-specific splenocyte proliferation. C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated with irradiated allogeneic BALB/c splenocytes (1×10^6 cells/ml) in 96-well microtiter plates for 5 days. Cultures were pulsed with $0.5 \mu\text{Ci}/\text{well}$ [methyl^3H]thymidine for the final 8 hr of the incubation period, and thymidine incorporation was determined by liquid scintillation spectrophotometry. Results are expressed as the mean cpm in quadruplicate samples \pm SD. Data are representative of three separate experiments. *, $P < 0.05$ (WT unmodified vs. WT IL-12-treated).

stimulated the secretion of IL-10 by alloantigen-stimulated WT splenocytes, but not IL-12R β 1^{-/-} cells (Table 1). Collectively, these data indicate that the β 1 subunit of IL-12R is required for the enhancement of several in vitro alloimmune responses by exogenous rIL-12, including increased alloantigen-stimulated T cell proliferation, and IFN- γ and IL-10 production.

Effects of Exogenous IL-12 on Alloimmune Responses in Vivo

Enhancement of serum IFN- γ by IL-12 treatment. To monitor the in situ effects of IL-12 treatment on IFN- γ production in cardiac allograft recipients, serum IFN- γ concentrations were measured on day 7 after transplantation (Fig. 3). In both WT and IL-12R β 1^{-/-} allograft recipients, serum IFN- γ was undetectable by ELISA on day 7 after transplantation. Treatment of WT recipients with rIL-12 markedly increased serum IFN- γ in three independent experiments; however, this treatment regimen had little effect on the concentration of serum IFN- γ in IL-12R β 1^{-/-} allograft recipients.

Effects of IL-12 on alloantigen-specific Th1 development in vivo. Splenocytes obtained from cardiac allograft recipients were restimulated in vitro with donor alloantigens and supernatant concentrations of IFN- γ were determined by ELISA. This assay detects in vivo primed Th1, in that splenocytes from naive, nontransplanted mice produce minimal or undetectable levels of IFN- γ under these conditions (15, 16, 24). Restimulation of splenocytes from unmodified IL-12R β 1^{-/-} allograft recipients with donor alloantigens resulted in the secretion of similar amounts of IFN- γ compared



Treatment In Vivo

FIGURE 3. Treatment of WT, but not IL-12R β 1^{-/-} allograft recipients with rIL-12 markedly increases serum IFN- γ . WT or IL-12R β 1^{-/-} C57BL/6 mice bearing BALB/c cardiac allografts were either left untreated or given daily intraperitoneal injections of $1.0 \mu\text{g}$ of rIL-12 on days 1–6 after transplantation. On day 7, blood obtained from allograft recipients was pooled and serum collected after centrifugation. Serum IFN- γ was determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples \pm SD. Data are representative of three independent experiments. ND indicates not detectable. ***, $P < 0.005$ (WT IL-12-treated vs. IL-12R β 1^{-/-} IL-12-treated).

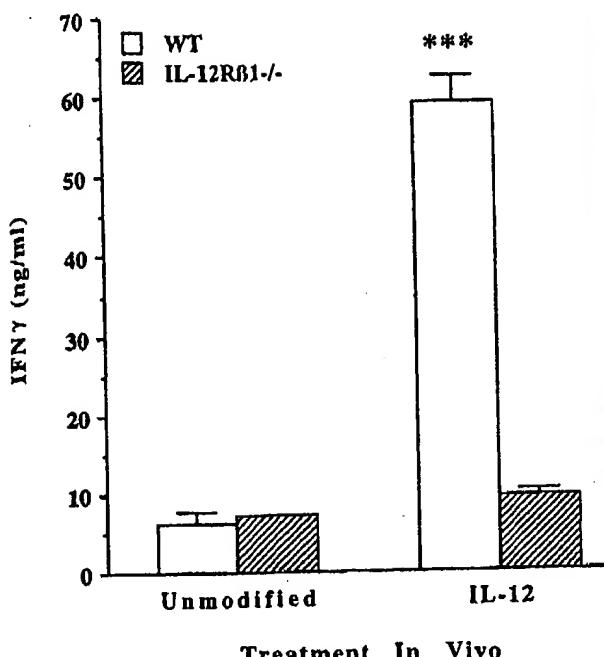
to that seen in WT recipients (IL-12R β 1^{-/-} = 7.16 ng/ml vs. WT = 6.24 ng/ml) (Fig. 4). Treatment of WT recipients with IL-12 resulted in a 10-fold increase in the production of IFN- γ (59.05 ng/ml). In contrast, IFN- γ production by splenocytes obtained from IL-12R β 1^{-/-} recipients treated with IL-12 in vivo was similar to untreated values (9.59 ng/ml), indicating that the β 1 subunit of IL-12R is required for IL-12-mediated enhancement of in vivo sensitization of IFN- γ -producing cells. Further, these results indicate that in vivo Th1 development can occur in a state of IL-12 unresponsiveness.

Effects of exogenous IL-12 on cardiac allograft rejection. As IL-12 treatment markedly enhanced Th1 responses in WT allograft recipients (Figs. 3 and 4), one might predict that IL-12 treatment would exacerbate allograft rejection. To test this possibility, cardiac allograft function was monitored by daily abdominal palpation in WT or IL-12R β 1^{-/-} allograft recipients bearing BALB/c hearts. Cardiac allograft recipients were either left untreated or injected once daily with $1.0 \mu\text{g}$ of rIL-12. Treatment of WT allograft recipients with this dose of rIL-12 ($n=10$) resulted in symptoms of cachexia including weight loss (mean decrease = $2.0 \pm 0.7 \text{ g}$ in 1 week), ruffed fur, hunched posture, and decreased activity. In contrast, IL-12R β 1^{-/-} allograft recipients exhibited no signs of IL-12-induced toxicity.

The mean cardiac allograft survival in unmodified WT recipients was approximately 8 days (data not shown; 15, 24).

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Treatment In Vivo

FIGURE 4. Enhancement of in vivo sensitization of IFN- γ -producing cells by IL-12 requires IL-12R β 1. WT or IL-12R β 1 $^{-/-}$ C57BL/6 cardiac allograft recipients were either left untreated or injected daily with 1.0 μ g of rIL-12 on days 1–6 after transplantation. To assess in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from cardiac allograft recipients were restimulated with irradiated BALB/c splenocytes (1×10^6 cells/ml). Supernatants were collected after 72 hr, and the concentration of IFN- γ was determined by ELISA. Results are expressed as the mean concentration of IFN- γ in triplicate samples \pm SD. Data are representative of three separate experiments. ***, P <0.005 (WT unmodified vs. WT IL-12-treated).

Cardiac allografts in IL-12R β 1 $^{-/-}$ recipients were rejected in a similar fashion to that seen in IL-12-deficient mice (15), in that grafts were uniformly rejected by day 7 ($n=8$). As expected, treatment of IL-12R β 1 $^{-/-}$ allograft recipients with rIL-12 had no effect on the tempo of allograft rejection ($n=6$). Interestingly, despite the overwhelming Th1 response induced by rIL-12 in WT allograft recipients (Figs. 3 and 4), treatment of these animals with rIL-12 did not appear to accelerate the tempo of graft rejection when compared to grafts of untreated WT recipients on day 7 after transplantation. For example, 7 of 10 (70%) allografts of WT recipients treated with rIL-12 were still functioning on day 7. A histologic evaluation of these grafts revealed similar parameters of early rejection compared to unmodified WT recipients. Specifically, histology was characterized by diffuse mononuclear cell infiltrates, viable myocytes as evidenced by visible nuclei, and relatively uninvolved vessels (Fig. 5). Hence, rIL-12 treatment did not accelerate the pathologic changes associated with acute rejection.

Phenotype of alloantigen-reactive Th1 in WT allograft recipients treated with rIL-12. To determine the phenotype of Th1 responsive to exogenous rIL-12, WT cardiac recipients were depleted in vivo of CD8+ T cells (Fig. 6). Splenocytes obtained from CD8 depleted cardiac allograft recipients produced markedly less IFN- γ upon in vitro restimulation with irradiated donor splenocytes (WT unmodified=6.15 ng/ml vs.

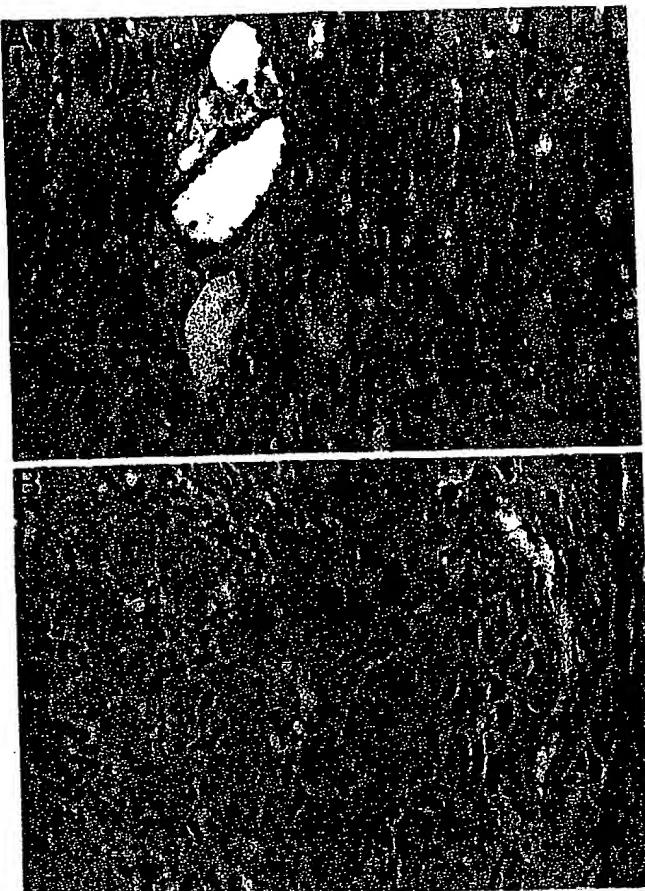


FIGURE 5. Exogenous IL-12 does not exacerbate cardiac allograft rejection. C57BL/6 WT recipients of BALB/c cardiac allografts were either left untreated or injected intraperitoneally with murine rIL-12 (1.0 μ g) on days 1–6 after transplantation. On day 7, allografts were harvested for histologic evaluation. (A) H&E-stained section of allografts from WT recipients left untreated (original magnification, $\times 400$). (B) H&E-stained section of allografts from WT recipients treated with rIL-12 (original magnification, $\times 400$). Note in both experimental groups moderate mononuclear cell infiltrates, and relative health of myocytes and vessels. These characteristics are associated with the early phase of acute rejection before onset of myocyte necrosis and vascular damage, which is observed on days 8 or 9 after transplantation. Results are representative of at least 10 individual transplants for each experimental group.

WT anti-CD8 mAb-treated=0.52 ng/ml). Similarly, Th1 that develop as a result of IL-12 stimulation in these experiments were predominantly CD8+ T cells (Fig. 6), as depletion of CD8 cells resulted in a reduction in IFN- γ production (WT IL-12-treated=35.89 ng/ml vs. WT IL-12 plus anti-CD8 mAb-treated=2.75 ng/ml).

IL-12 Treatment (1.0 μ g/Day) Inhibits Alloantibody Responses

Given our findings that treatment of WT cardiac allograft recipients with rIL-12 resulted in significant augmentation of serum IFN- γ (Fig. 3) and in vivo priming of Th1 (Fig. 4), one would predict that IL-12 treatment should drive IgG2a alloantibody production. To test this possibility, sera alloantibody production was assessed on day 7 after transplan-

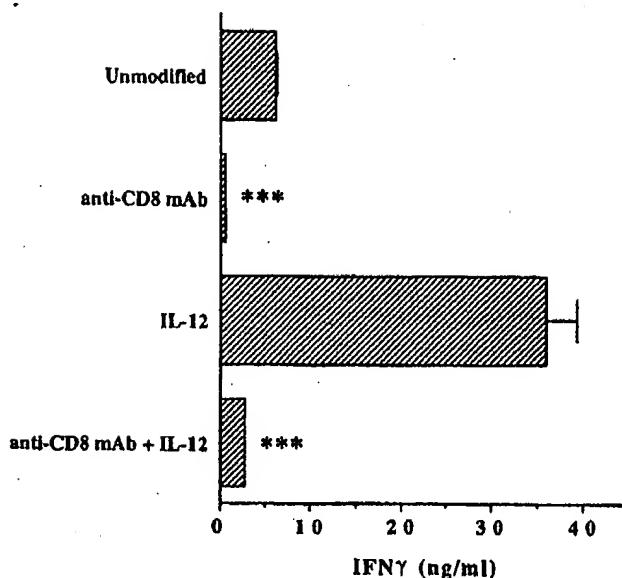


FIGURE 6. Alloantigen-specific Th1 responding to exogenous rIL-12 are CD8+ T cells. In these experiments, splenocytes were obtained from cardiac allograft recipients either left untreated or treated with anti-CD8 mAb, rIL-12, or a combination of anti-CD8 mAb plus rIL-12. Th1 function was assessed by IFN- γ production after a 72-hr restimulation of recipient's splenocytes with irradiated BALB/c splenocytes. Results are expressed as the mean concentration of IFN- γ in triplicate samples \pm SD. Data are representative of three separate experiments. ***, P<0.005 (WT unmodified vs. WT anti-CD8 mAb-treated; WT IL-12-treated vs. WT IL-12 plus anti-CD8 mAb-treated).

tion in WT cardiac allograft recipients either left unmodified or treated once daily with 1.0 μ g of rIL-12. In these experiments, sera IgG2a was undetectable in rIL-12-treated WT allograft recipients at this time point (data not shown). Further, treatment of WT recipients with rIL-12 resulted in reduced sera IgM alloantibody in three independent experiments, compared to untreated WT recipients (Table 2). This observation indicates that high doses of rIL-12 inhibit, rather than enhance, alloantibody production in this model. In contrast, treatment of IL-12R β 1 $^{-/-}$ allograft recipients with

rIL-12 resulted in a slight increase in sera IgM compared to unmodified knockout recipients.

Effects of Low-dose IL-12 (0.1 μ g/Day) on Alloantibody Responses

The reduction in sera IgM in WT allograft recipients after rIL-12 (1.0 μ g/day) treatment (Table 2) may have been caused by anti-proliferative or toxic effects on B cell function caused by rIL-12 and/or IFN- γ . To test this possibility, WT cardiac allograft recipients were treated once daily with 0.1 μ g of rIL-12 and sera alloantibody levels were assessed on day 7 or 8 after transplantation. No alterations in the pathology of allograft rejection was observed in recipients treated with 0.1 μ g of rIL-12 compared to 1.0 μ g (data not shown). Further, treatment of WT allograft recipients with 0.1 μ g of rIL-12 resulted in significantly less IL-12-induced toxicity, serum IFN- γ concentrations in these animals were undetectable by ELISA, and in vivo Th1 sensitization was similar to untreated allograft recipients (data not shown). These results suggested that the 0.1- μ g dose of rIL-12 was ineffective in vivo. However, unlike the higher dose of rIL-12 (1.0 μ g), treatment of WT cardiac allograft recipients with 0.1 μ g of rIL-12 resulted in an increase in sera IgG2a, but not IgG1 at both days 7 and 8 after transplantation (Table 3). Further, IL-12 treatment augmented sera IgM levels at both time points.

DISCUSSION

Bioactive IL-12 exists as a p70 heterodimer composed of p35 and p40 subunits (28, 29). Each subunit of IL-12 interacts with a distinct component of the IL-12R: p40 binds to IL-12R β 1 and p35 interacts with IL-12R β 2 (19, 20). Both receptor subunits are associated with members of the Janus kinase family (30), and therefore may facilitate IL-12-mediated signal transduction. However, most of the attention has been given to IL-12R β 2, which associates with JAK2 (30). For example, recent evidence supports a requirement for IL-12R β 2 expression in IL-12-induced phosphorylation of Stat4 (21, 22). Further, these studies revealed the importance of the β 2 subunit of IL-12R by demonstrating that the unresponsiveness of Th2 to IL-12 in both human (21) and mouse (22) is a result of loss of IL-12R β 2 expression by these cells. These results indicate that the binding of the p40 subunit of

TABLE 2. High-dose rIL-12 inhibits IgM production in vivo^a

	Serum IgM (mean channel fluorescence)	
	Unmodified	IL-12 Treatment
WT		
Experiment 1	37.18	9.85
Experiment 2	33.97	11.39
Experiment 3	78.51	27.37
IL-12R β 1 $^{-/-}$		
Experiment 1	17.36	31.52
Experiment 2	33.83	43.64
Experiment 3	21.67	35.96

^a Serum was obtained on day 7 after transplantation from WT or IL-12R β 1 $^{-/-}$ cardiac allograft recipients. Animals were either left untreated or injected with 1 μ g of rIL-12 once daily. Anti-BALB/c IgM was assessed by flow cytometry using P815 (H2^d) target cells as described under Materials and Methods. Data are reported as the mean channel fluorescence and represent three separate experiments for each group.

TABLE 3. Low-dose rIL-12 augments IgM and IgG2a production in vivo^a

	Mean channel fluorescence		
	IgM	IgG1	IgG2a
Experiment 1 (day 7)			
Unmodified	11.90	2.06	2.58
IL-12	28.94	2.67	12.79
Experiment 2 (day 8)			
Unmodified	31.54	8.53	16.31
IL-12	46.57	9.72	49.00

^a Serum was obtained on day 7 or 8 after transplantation from cardiac allograft recipients either left untreated or injected with 0.1 μ g of rIL-12 once daily. Isotype-specific anti-BALB/c alloantibody was assessed by flow cytometry using P815 (H2^d) target cells as described under Materials and Methods. Data are reported as the mean channel fluorescence. Mean channel fluorescence for isotype controls were 1.65 (IgM), 1.67 (IgG1), and 1.57 (IgG2a).

IL-12 to IL-12R β 1 is not sufficient to mediate the bioactivity of heterodimer IL-12. However, we have reported that p40 promotes alloantigen-specific CD8+ Th1 development in the absence of heterodimer IL-12 (15). This observation suggests that IL-12 p40 mediates its stimulatory effect through IL-12R β 1 alone, or that IL-12R β 1 associates with a yet unidentified component of IL-12R on CD8+ T cells. These possibilities have not been tested. Additional data are emerging that support a biologic role of p40 interacting with IL-12R β 1 (1). Specifically, p35 knockout mice, which are capable of producing p40 in levels similar to WT mice (31), are less susceptible to infection with *Listeria* and *Cryptococcus neoformas* compared to p40 knockout mice. Hence, one goal of the current study was to assess the role of IL-12R β 1 in alloimmune responses both in vitro and in vivo.

IL-12 is a potent stimulator of in vitro alloantigen-specific Th1 development, in that the addition of IL-12 to MLC consisting of WT responder splenocytes resulted in a 10-fold or greater increase in IFN- γ production (Table 1; 15). Exogenous rIL-12 also markedly augments in vitro Th1 development in mice that are deficient in p35, p40 (15) or both subunits of IL-12 (JR Piccotti and DK Bishop, unpublished observations), indicating that T cells of these mice are equipped with a functional IL-12R. In contrast, IL-12 did not alter MLC IFN- γ production by splenocytes of IL-12R β 1 $^{-/-}$ mice (Table 1). This result illustrates the requirement of β 1 subunit of IL-12R for IL-12-driven Th1 differentiation in vitro. It should be noted that, although IFN- γ production by IL-12R β 1 $^{-/-}$ splenocytes in MLC was reduced compared to WT values (Table 1), this cytokine was readily detectable by ELISA, suggesting that IL-12 is not an absolute requirement for in vitro Th1 responses.

IL-12 is also a key cytokine involved in promoting cell-mediated immune responses in vivo (1, 2). However, what role IL-12 plays in transplant rejection remains unclear. It has been reported that IL-12 has a central role in the progression of acute graft-versus-host disease (GVHD) in mice (6, 32). In these studies, neutralizing IL-12 with a polyclonal anti-IL-12 antibody results in the amelioration of acute GVHD (32) and, conversely, treatment with exogenous IL-12 converts chronic GVHD into exacerbated acute GVHD (6, 32). Further, Williamson et al. (33) have reported that neutralizing IL-12 during the inductive phase of GVHD results in a Th1 to Th2 shift evidenced by a reduction in IFN- γ and enhancement of IL-5 and IL-10 production by Con A-stimulated splenocytes. In contrast to these findings, neutralizing IL-12 in mouse vascularized cardiac allograft recipients promotes intragraft Th2 cytokine (IL-4 and IL-10) gene expression; however, these grafts are rejected in an accelerated fashion compared to untreated recipients (16). Importantly, in vivo Th1 priming is not inhibited by IL-12 neutralization, indicating that Th1 development can occur independent of IL-12 (16). This possibility is further supported by the observation that splenocytes of IL-12R β 1 $^{-/-}$ allograft recipients produce similar concentrations of IFN- γ upon restimulation with donor splenocytes compared to WT recipients (Fig. 4). It does not appear that Th1 development in IL-12R β 1 $^{-/-}$ mice is a result of the interaction of endogenous IL-12 with the low-affinity IL-12R β 2, as treatment of these animals with rIL-12 did not augment in vivo priming of IFN- γ -producing cells (Figs. 3 and 4).

A second hypothesis tested in the current study was treatment of cardiac allograft recipients with IL-12 would accelerate the rejection process as a result of exacerbated Th1-driven immune responses. Administration of exogenous rIL-12 significantly augmented in vivo sensitization of IFN- γ -producing cells in WT cardiac allograft recipients, as evidenced by increased sera IFN- γ (Fig. 3) and enhanced production of IFN- γ by splenocytes after restimulation with donor alloantigens in vitro (Fig. 4). However, this fulminate Th1 response in vivo did not result in anticipated acceleration of graft rejection when compared to untreated control recipients (Fig. 5). It is possible that induction of high systemic levels of IFN- γ results in an inhibition of immune response as a result of IFN- γ 's anti-proliferative properties on effector cell development (34). However, graft survival was not prolonged after rIL-12 treatment in the current study. This observation questions the overall importance of Th1 responses in this experimental model, and suggests that the magnitude of Th1-driven alloimmune response may not correlate directly to the severity of graft rejection. Indeed, Th2-driven immune responses are emerging as potential effector cells of rejection in both human and experimental transplantation (reviewed in 35).

Finally, we examined the influence of rIL-12 administration on allospecific B cell function. In an experimental system in which PVG.RT1^a congenic rats were immunized with an isolated alloantigen, Gracie et al. (36) reported that treatment with murine rIL-12 (1.0 μ g/day for 5 days) after alloimmunization augments levels of allospecific IgG2b and IgG2c, while decreasing IgG1. The authors demonstrated that co-administration of neutralizing anti-IFN- γ mAb abrogated this response, indicating that the enhancement of B cell function by IL-12 was dependent on IFN- γ . When adjusted for body weight, this dose of rIL-12 in the rat is comparable to our 0.1- μ g dose in the mouse. In the current study, treatment of WT cardiac allograft recipients with 0.1 μ g of rIL-12/day for 6 days increased the level of sera IgG2a compared to untreated recipients on day 7 and 8 after transplantation (Table 3). However, administration of 1.0 μ g of rIL-12/day reduced allospecific B cell function, indicated by a decrease in sera IgM (Table 2) and absence of isotype switch to IgG2a. These observations suggest a biphasic response to IL-12 treatment in WT mouse cardiac allograft recipients likely dependent on the concentration of IFN- γ .

In summary, this study illustrates that the β 1 subunit of mouse IL-12R is critical for IL-12-driven alloimmune responses both in vitro and in vivo, and that IL-12R β 2 alone does not transduce IL-12 signaling. These observations are supported by recent reports, which have shown that humans deficient in IL-12R β 1 exhibit severe impairment in their resistance to infections as a result of intracellular pathogens (37, 38). The generation of mice deficient in IL-12R β 2 will provide an important animal model to evaluate whether β 1 subunit of IL-12R alone conveys IL-12 responsiveness in vivo. Specifically, these mice would be useful in determining the mechanism by which p40 subunit of IL-12 enhances CD8+ Th1 development (15, 16). Finally, this study questions the importance of Th1-driven alloimmune responses in cardiac allograft rejection, as exacerbated Th1 responses induced by IL-12 failed to accelerate graft rejection in this model.

Acknowledgments. The authors thank Drs. Jeanne Magram and Maurice K. Gately for generously providing IL-12R β 1 $^{-/-}$ mice and murine rIL-12, and for their helpful discussions. The authors also thank Brooke Schuemann for technical support.

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Received 18 August 1998.

Accepted 4 December 1998.

Zinc Inhibits the Mixed Lymphocyte Culture

CATHRIN A CAMPO,¹ NELE WELLINGHAUSEN,²
CHRISTIAN FABER,¹ ANDREA FISCHER,¹ AND LOTHAR RINK^{1,*}

¹Institute of Immunology and Transfusion Medicine,
University of Lübeck School of Medicine, Lübeck, Germany;
and ²Section of Infectious Diseases and Clinical Immunology,
University of Ulm, Ulm, Germany

Received June 13, 2000; Accepted July 10, 2000

ABSTRACT

The mixed lymphocyte culture (MLC) is an established clinical method for bone marrow transplantation, as it serves as an *in vitro* model for allogenic reaction and transplantation. We previously showed that cytokine release into the supernatant is a more specific and sensitive parameter for cross-reactivity in the MLC than the common measurement of cell proliferation. Therefore we tried to find an inhibitor of the MLC *in vitro* with the least side effects *in vivo*, measuring interferon (IFN)- γ as one of the most important cytokines in posttransplant medicine. Earlier studies showed that zinc is an important trace element for immune function with both stimulatory and inhibitory effects on immune cells. We found that slightly elevated zinc concentrations (three to four times the physiological level), which do not decrease T-cell proliferation *in vitro* nor produce immunosuppressive effects *in vivo*, suppress alloreactivity in the mixed lymphocyte culture. In this report we analyzed the mechanism whereby zinc influences the MLC to possibly find a nontoxic way of immunosuppression.

Index Entries: Mixed lymphocyte culture (MLC); mixed lymphocyte reaction (MLR); trace elements; zinc.

INTRODUCTION

The mixed lymphocyte culture (MLC) is a well-established and important tool for determination of compatibility between host and donor

*Author to whom all correspondence and reprint requests should be addressed.

in transplantation medicine, as it serves as an in vitro model for allogenic reaction (1,2). It is common to measure T-cell proliferation in the MLC, but it was recently shown that cytokines are more specific and sensitive parameters for the prediction of a possible graft rejection, as they play a critical role in the posttransplant response (3-5). The TH1-cytokine interferon- γ (IFN- γ) was identified as the most important factor within the cytokine cascade in the MLC (6). It is known to induce cytotoxic T-lymphocytes (CTL) (7) by enhancing the expression of both major histocompatibility complex (MHC) class I and MHC class II molecules (8). The IFN- γ response mainly depends on HLA-DR differences and it therefore well represents reactivity between two individuals in the MLC (9).

In transplantation medicine, cyclosporin A, FK506, and other substances are used to prevent graft rejection. In vitro experiments revealed an inhibition of the MLC (10), but, unfortunately, all of these immunosuppressants show a wide range of toxicities in vivo, such as nephrotoxicity, neurotoxicity, and, probably, carcinogenicity (11-13). As we are beginning to understand the molecular mechanisms of cyclosporin A and FK506 function better and better, one of the major aims is to find similar substances with less toxicity.

Zinc within the physiological range (12-16 μM) is an important trace element for immune function (14). Zinc deficiency in vivo could be linked to various clinical symptoms such as impaired immune response with regard to decrease in number, differentiation, and function of T-lymphocytes and natural killer (NK) cells as well as decreased activation of monocytes and phagocytosis by macrophages, resulting in a high incidence of bacterial, viral and fungal infections. These symptoms, in the most severe form shown in the hereditary disease acrodermatitis enteropathica caused by malabsorption of zinc, are completely reversible after adequate substitution of zinc (15). On the other hand, high concentrations of zinc (about eight times the physiological level) led to cytotoxic effects with impairment of all T-cell functions, and inhibition of monokine induction by superantigens such as zinc is also important for the binding of some bacterial superantigens to the β -chain of the MHC class II molecule (16,17). Optimal immune-cell function hence requires a well-balanced zinc level.

In the following study, we investigated whether zinc is able to impair alloreactivity in the MLC at concentrations with neither cytotoxic effects in vitro nor toxic side effects in vivo.

MATERIALS AND METHODS

Preparation of Lymphocyte Cultures

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by density centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany), washed twice with phosphate-buffered saline (PBS, Gibco, Berlin, Germany) and resuspended in RPMI-1640 medium (Biochrom) supplemented with 10% heat-inactivated fetal calf

serum (FCS, low endotoxin, myoclone quality; Life Technologies, Eggenstein, Germany), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all obtained from Biochrom, Berlin, Germany). The cells were adjusted to a final concentration of 2×10^6 cells/mL. Equal volumes of cell suspensions of two donors were seeded in samples to a final volume of 1 mL into pyrogen-free 24-well culture plates (Falcon, Heidelberg, Germany). For controls, 1 mL of the adjusted cell suspension was cultured separately. The cultures were incubated for 5 d at 37°C in a 5% humidified CO₂ atmosphere after addition of the appropriate amount of zinc.

Zinc Preparations

Zinc sulfate (Sigma, Delsenhofen, Germany) was dissolved in sterile water to achieve a zinc stock solution of 10 mM. This solution was further diluted in unsupplemented protein-free medium (PFM, Ultradoma, BioWhittaker) at a ratio of 1 to 2 and then sterile filtered. To achieve the final concentrations, PFM was used. The zinc solution was added to the cultures in a volume of 10% of the final culture volume.

Determination of Cytokines

The culture supernatants were harvested after 5 d and stored at -80°C. The quantification of the cytokine release into the supernatant was performed by enzyme-linked immunosorbent assay (ELISA) technique (for IFN-γ provided by Bender Med Systems, Vienna, Austria). Results were measured in picograms per milliliter at 450 nm using an ELISA plate reader (Anthos Labtec, Salzburg, Austria).

Flow Cytometry

Propidium Iodide (PI) staining was performed by using a stock solution of 1 mg/mL (PI, Sigma). Cells (1×10^6 /mL) were incubated with 10 µL of PI stock solution for 20–30 min to allow intercalation of PI in double-stranded DNA. Finally, PI staining was measured at a wavelength of 620 nm in a flow cytometer (Coulter, Krefeld, Germany).

Statistical Analysis

The results are expressed as median values. The significance is taken by Student's *t*-test analysis.

RESULTS

Influence of Zinc on Mixed Lymphocyte Cultures

We harvested the supernatants of zinc-supplemented mixed lymphocyte cultures (MLC) on d 5, proven to be the maximum of the IFN-γ secretion (18). Analyzing IFN-γ release in 20 MLC experiments supplemented with different concentrations of zinc, we found expected amounts of IFN-

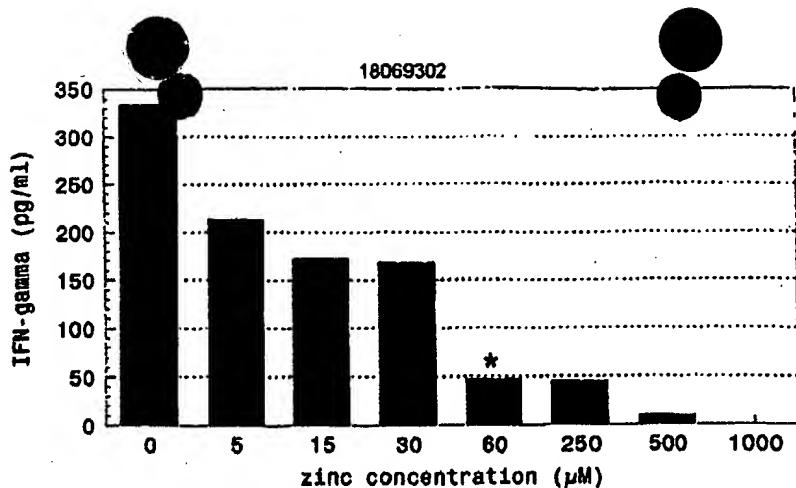


Fig. 1. IFN- γ secretion in the MLC after zinc supplementation. Zinc concentrations up to 1 mM were added to mixed lymphocyte cultures; controls remained unsupplemented. Secretion of IFN- γ in the culture supernatants was determined by ELISA after 5 d of culture. Median values of $n = 20$ experiments are expressed in picograms per milliliter. Significance was calculated by the Student's t -test (* $p = 0.0017$).

γ (334 pg/mL) in the supernatant of control MLC without zinc addition, whereas increasing zinc concentrations led to a dose-dependent reduction of the IFN- γ level. At 60 μM , the IFN- γ production was significantly diminished (48 pg/mL, $p = 0.0017$); at 500 μM , no IFN- γ was detectable (Fig. 1).

In order to prove our hypothesis that this result—that zinc concentrations of 60 μM inhibit the MLC—was the result of a specific effect in the MLC and not to a loss of T-cell vitality, we added zinc concentrations of up to 5 mM to PBMC and measured cell viability by flow cytometry after an incubation time of 48 h. Figure 2 shows that 93.2% of the cells are still vital after addition of 50 μM zinc and 92.3% with medium supplementation of 100 μM zinc compared to controls without zinc addition with 91.3% viability. Zinc concentrations as high as 250 μM causes a reduction of cell survival of 33% (Fig. 2).

For further analysis of possible mechanisms responsible for this inhibition, we preincubated PBMC with 50 μM zinc for 20 min and then cocultured these two populations in the MLC. The results reveal a marked influence of the point of time at which zinc is added to the culture: Preincubation of PBMC led to a greater reduction of IFN- γ than simultaneous zinc supplementation to the MLC (Fig. 3).

DISCUSSION

The human mixed lymphocyte culture (MLC) is an important method to test donor-recipient compatibility in bone marrow transplantation. It could be shown that cytokine release, especially IFN- γ , has a very good predictive value with regard to the transplantation outcome (3), as cytokines play a major role in the generation of an alloreactive immune response and

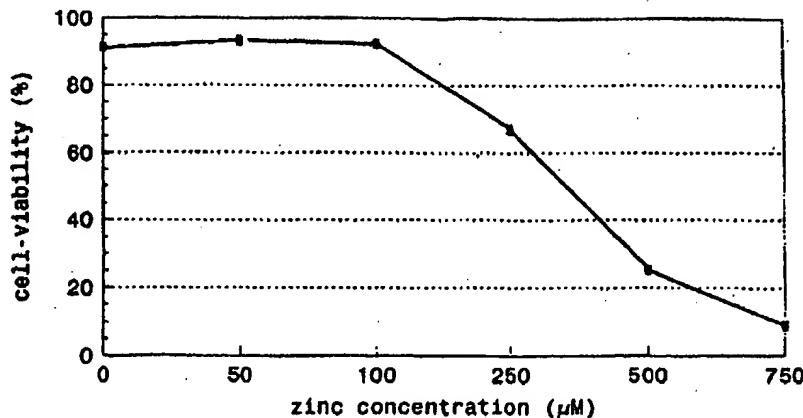


Fig. 2. Viability of PBMC after zinc supplementation. Zinc concentrations of up to 750 μM were added to unstimulated PBMC; controls remained unsupplemented. Cell viability was determined by flow cytometry after an incubation time of 48 h. One representative experiment is shown, values are expressed in percent of the total cell population.

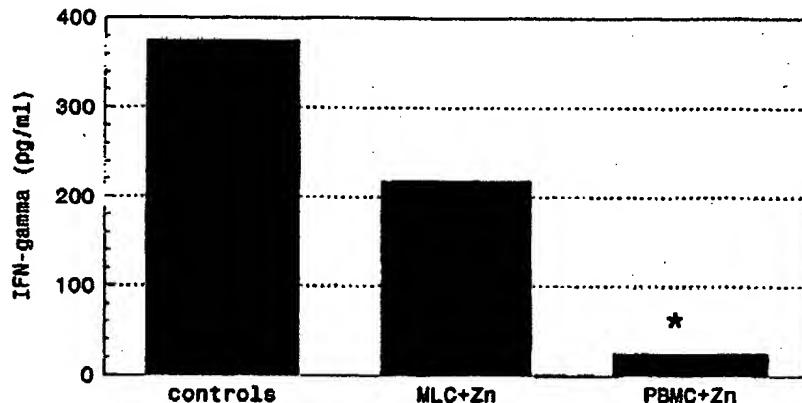


Fig. 3. Effect of preincubation of PBMC with zinc. Zinc in a concentration of 50 μM was added to the MLC simultaneously (MLC + Zn); PBMC was preincubated with 50 μM zinc for 20 min and then cocultured in a MLC (PBMC + Zn); controls remained unsupplemented. Medians of $n = 10$ experiments are presented. Significance was calculated by the Student's *t*-test (* $p = 0.01$).

for the induction of graft rejection *in vivo* (4,5). Taking this *in vitro* model, it has always been the aim to inhibit proliferation of immune cells in order to find a way to prevent graft rejection in transplantation medicine. Landolfo et al. inhibited T-cell reactivity by the addition of anti-IFN- γ both *in vitro* and *in vivo* (19); others showed a reduced graft rejection rate by repeated injections of monoclonal anti-IFN- γ antibodies in a skin-, heart-, or pancreas-tissue transplantation situation (20–22).

In vivo substances like cyclosporin A or FK506 are broadly applied, as they are capable of prolonging graft survival. In vitro, they show an inhibitory effect on T-cell proliferation in the MLC (10). Yet, all of these therapeutical agents cause major side effects (e.g., nephrotoxicity, neurotoxicity, and others), which lead to a limitation of their use (11-13).

Zinc is an essential trace element with great influence on immune function. The physiological plasma level of zinc ranges from 12 to 16 μM . In our study, we applied zinc concentrations up to 100 μM , which can be reached by pharmacological application of zinc in vivo without causing side effects (23).

We found that zinc concentrations of 60 μM , four times the physiological level, inhibit alloreactivity in the MLC. It is unlikely that the reduction of IFN- γ is the result of a loss of T-cell activity, as it could be shown earlier that T-cells are still able to proliferate in medium supplemented with zinc concentrations as high as 100 μM (24). Furthermore, we analyzed the viability of the PBMC by flow cytometry, showing that a concentration of 250 μM is required to reduce cell viability by 33% (Fig. 2).

Increased zinc levels of over 100 μM cause unstimulated human PBMC to release cytokines (25). This stimulatory effect of zinc is only seen in the presence of accessory cells, especially monocytes, as mostly IL-1 proved to be an essential cosignal for T-cell activation by zinc. Higher concentrations of zinc impair all T-cell and monocyte function by inhibition of the IL-1 receptor type I-associated protein kinase (IRAK), thus blocking the intracellular-signal transduction pathway at a very early stage (24).

In our study, we applied zinc in concentrations that neither show cytotoxic effects nor reach stimulatory level. Therefore, there seems to be a specific effect of zinc on the responding T-cells in the MLC.

The results of earlier studies proposed an oligoclonal pattern of T-cell stimulation in the MLC similar to T-cell activation by superantigens (3). Furthermore, a highly altered V β repertoire of T-cells infiltrating long-term rejected kidney allografts were described (26). Superantigens bind directly and partially with high affinity to major histocompatibility complex (MHC)-class II proteins, especially to HLA-DR. T-Cell activation is achieved by the formation of a complex of the V β -chain of the T-cell receptor (TCR), the MHC molecule, and the superantigen. This binding is regulated by zinc, as zinc itself does not interact with the MHC molecule directly (27). We previously showed that the HLA-DR and HLA-DQ-molecules have the greatest influence on cytokine release in the MLC and thus on the outcome of a transplantation in vivo (9).

There are two main possible explanations for the phenomenon described. First, zinc in the applied concentration could saturate the MHC and, therefore, prevent a binding between TCR and MHC. In order to prove this hypothesis, we preincubated PBMC with zinc and then cocultured these populations. If an extracellular mechanism were actually responsible for the inhibition of the MLC, we would expect no significant difference in IFN- γ secretion in either setting. Preincubation of PBMC

resulted in a markedly lower IFN- γ secretion than the culture of two PBMC populations with simultaneous zinc supplementation to the MLC (Fig. 3), so that it seems more likely that zinc interferes with the intracellular signal transduction in the MLC. Therefore, zinc may regulate the alloreactivity of T-cells and might be an explanation for increased preterm delivery and abortion in zinc-deficient pregnant women (28,29). As mentioned earlier, higher concentrations of zinc are able to block the intracellular signal transduction pathway by inhibition of IRAK. We propose that the stimulation of T-cells by an HLA-different cell population can be blocked by zinc via specific inhibition of phosphorylation processes, leading to a diminished signal transduction in the cell. This results, among other things, in reduced secretion of cytokines, which should lead to less graft rejection *in vivo*. Various protein kinases such as cAMP- and cGMP-dependent protein kinases as well as protein tyrosine kinases are involved in zinc-induced cell stimulation and zinc also influences gene expression of different immunologically relevant transcription factors such as nuclear factor (NF)- κ B and metallothionein transcription factor (MTF-1) as well as others. Which alteration of signal transduction zinc exactly inhibits the MLC remains the subject of further investigation. Because the MLC is inhibited by very low zinc concentrations, this inhibitory effect seems to be a specific pathway.

In conclusion, zinc could become an immunosuppressant in transplantation medicine without toxic side effects, which still leaves the immune system with the ability for phagocytosis. The infection rate will therefore be reduced compared to current immunosuppression. However, this has yet to be proven in *in vivo* transplantation models.

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Differential effects of IL-12 on the generation of alloreactive CTL mediated by murine and human dendritic cells: a critical role for nitric oxide

Yasuhiro Nishioka,* Hua Wen,* Kayo Mitani,* Paul D. Robbins,† Michael T. Lotze,‡,§
Saburo Sone,* and Hideaki Tahara§

*Third Department of Internal Medicine, University of Tokushima School of Medicine, Japan;
Departments of †Surgery and ‡Molecular Genetics and Biochemistry, School of Medicine,
University of Pittsburgh Cancer Institute, Pennsylvania; and §Department of Surgery,
Institute of Medical Science, School of Medicine, University of Tokyo, Japan

Abstract: We examined the mechanisms involved in interleukin (IL)-12-mediated suppression of cellular immunity in mice using allogeneic mixed leukocyte reaction (MLR) stimulated by dendritic cells (DCs) *in vitro* and compared the effect of IL-12 on MLR in mice and humans. Although IL-12 stimulated human MLR, the addition of IL-12 or interferon- γ (IFN- γ) resulted in a dose-dependent suppression of MLR in mice. The treatment with L-N-monomethyl-L-arginine (L-NMMA) completely abrogated IL-12- and IFN- γ -mediated suppression of MLR in mice. Furthermore, IL-12 enhanced the alloreactive cytolytic T lymphocyte (CTL) induction in human MLR, whereas the addition of L-NMMA was required to generate alloreactive CTLs in the presence of IL-12 in mice. Nitric oxide (NO) was detected only in mouse MLR. Murine DCs could produce NO, but neither human CD34 $^{+}$ cell- nor monocyte-derived DCs produced a detectable amount of NO. These results suggest that NO produced by DCs might play an important role in IL-12-mediated immune suppression in mice but not in humans. *J. Leukoc. Biol.* 73: 621–629; 2003.

Key Words: MLR · IFN- γ · L-NMMA

INTRODUCTION

Interleukin (IL)-12 is a heterodimeric cytokine produced by dendritic cells (DCs), macrophages, polymorphonuclear leukocytes, and keratinocytes [1]. Pleiotropic effects on the host immune system, including activation of natural killer (NK) cells and cytolytic T lymphocytes (CTLs), induction of interferon- γ (IFN- γ) production and T helper cell type 1 (Th1) differentiation, and antiangiogenic effects [1–4], have been reported. Exogenous IL-12 is effective for infectious and malignant diseases [1–6]. Furthermore, IL-12 gene transduction into tumors strongly suppressed the growth of various tumors *in vivo* [7, 8], whereas the mechanisms involved in the antitumor

effects of IL-12 are still unclear as a result of the complexity of the biological effects of IL-12.

Conversely, the unexpected effects of IL-12 on the cellular immune responses have been reported in mice. Noguchi et al. [9] showed that administration of a low dose of IL-12 (1 ng/mouse) enhanced CTL activity specific for the mutated p53 peptide (234CM) and regressed the MethA tumor expressing it, whereas a high dose of IL-12 (100 ng/mouse) suppressed 234CM-specific CTL generation. Martinotti et al. [8] reported that a CD8 $^{+}$ T cell-mediated *in vivo*-immune response against IL-12-transduced colon cancer cells was observed only when CD4 $^{+}$ T cells were abrogated. Furthermore, Orange et al. [10] reported that administration of IL-12 decreased antiviral CTL activity in the lymphocytic choriomeningitis virus (LCMV) infection model. The report presented by Piccotti et al. [11] demonstrated that IL-12 antagonism using anti-IL-12 antibodies (Ab) or an IL-12 p40 homodimer exacerbated cardiac allograft rejection in mice. Recently, Kurzawa et al. [12] clearly demonstrated the suppressive effects of IL-12 on cellular immune responses using syngeneic and allogeneic murine tumor systems. Further studies reported by Koblish et al. [13] clarified that nitric oxide (NO) produced by macrophages was responsible for IL-12-mediated immune suppression *in vitro* and *in vivo*. On the basis of these results, they suggested the monitoring of suppressive effects of IL-12 when used for malignant and infectious diseases in humans.

DCs are known to be specialized antigen-presenting cells (APCs), which exist in virtually every tissue, capture antigens *in situ*, and migrate to lymphoid organs to activate naive T cells and play a key role in the induction of primary immune responses [14]. As murine DCs are also known to be the producer of NO [15], we examined whether NO derived from DCs is a suppressive factor for the cellular immune responses in allogeneic mixed leukocyte reaction (MLR) stimulated by IL-12 in mice and humans. It is interesting that a high dose of

Correspondence: Yasuhiro Nishioka, Third Department of Internal Medicine, University of Tokushima School of Medicine, Kuramoto-cho 3, Tokushima 770-8503, Japan. E-mail: yasuhiro@clin.med.tokushima-u.ac.jp

Received April 25, 2002; revised December 24, 2002; accepted January 23, 2003; doi: 10.1189/jlb.0402205.

IL-12 suppressed alloreactive proliferation of T cells in mice but enhanced it in humans. Furthermore, we found the high production of NO by murine DCs, whereas neither human CD34⁺ progenitor cell- nor monocyte-derived DCs produce a significant amount of NO even after stimulation with IFN- γ and/or lipopolysaccharide (LPS) as well as MLR. These results suggest that immune responses mediated by IL-12 in mice are, at least in part, different from that in humans and that the suppressive effect of IL-12 on cellular immune responses might not be the case in humans.

MATERIALS AND METHODS

Reagents

The culture media was RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 μ g/ml streptomycin, 100 U/ml penicillin, and 5.5×10^{-5} M 2-mercaptoethanol (all from Life Technologies, Grand Island, NY), referred to henceforth as complete medium (CM). The recombinant mouse and human (rh)IL-12 was kindly provided by Dr. Maurice Cately (Hoffmann-LaRoche, Nutley, NJ). The rhIFN- γ was purchased from Boehringer Mannheim (Indianapolis, IN). Specific activities were $>5.0 \times 10^6$ U/mg and $>2.0 \times 10^7$ U/mg, respectively. N⁶-monomethyl-L-arginine (L-NMMA) and NO-generating agent S-nitroso-N-acetyl-penicillamine (SNAP) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines and mouse strains

EL-4 and P815 were generous gifts of William H. Chambers (University of Pittsburgh, PA). These cell lines were maintained in CM as described previously [7].

Female 6- to 8-week-old C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). Female 6- to 8-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These animals were used for all experiments at the age of 8–10 weeks old [7].

Culture of mouse bone marrow (BM)-derived DCs

BM-DC culture was obtained using methods described previously [16–19]. Briefly, murine BM cells were harvested from the femur and tibia of killed mice. Contaminating erythrocytes were lysed with 0.82 M NH₄Cl buffer, and lymphocytes were depleted with a cocktail of antibodies (RA3-3A1/6.1, anti-B220; 2.43, anti-IgM 2; and GK1.5, anti-ICR14; all from American Type Culture Collection, Manassas, VA) and rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY) on day 0. These cells were cultured overnight in CM to remove the adherent macrophages, and then nonadherent cells were placed in fresh CM containing murine granulocyte macrophage-colony stimulating factor (GM-CSF; 1000 U/ml) and rHuIL-4 (1000 U/ml; Shering-Plough, Kenilworth, NJ; DC media) on day 1. Cells were generally harvested on day 6. BM-DCs were defined by morphology, phenotype, and strong mixed lymphocyte reaction-stimulating activity. Phenotypic analysis by flow cytometry showed a high expression of CD11b, CD11c, CD80, CD86, as well as major histocompatibility complex class I and class II in the majority of the cultured cells (60–95%) [19]. These DCs were used for the subsequent experiments as immature DCs.

Flow cytometry

For phenotypic analysis of DCs, phycoerythrin- or fluorescein isothiocyanate-conjugated monoclonal Ab (mAb) against murine and human cell-surface molecules [CD11b, CD11c, CD80, CD86, Gr-1, H-2K^b, I-A^b (murine), CD1a, CD14, CD40, CD54, CD80, CD86, human leukocyte antigen (HLA)-ABC, HLA-DR, and appropriate isotype controls, all from PharMingen, San Diego, CA] were used. DCs were stained and analyzed on a FACScan using LYSIS II software (Becton Dickinson, Mountain View, CA) as described previously [19].

Purification of human CD34⁺ progenitor cells from BM

Human BM cells were harvested after informed consent. Mononuclear cells (MNC) were purified from human BM cells using density centrifugation with lymphocyte separation medium (LSM; Litton Bionetics, Kensington, MD). These cells were applied for immunoaffinity column (CellPro, Inc., Bothell, WA) after lysis of erythrocytes to purify CD34⁺ cells [20]. Purity of CD34⁺ cells was more than 85%, as determined by flow cytometry. These CD34⁺ cells were frozen down in liquid nitrogen until used for DC generation.

Generation of human CD34⁺ progenitor cell-derived DCs

On day 0, CD34⁺ cells (5×10^5 cells) were thawed and cultured in six-well plates with cRPMI 1640 with GM-CSF (500 U/ml), tumor necrosis factor α (50 ng/ml), stem cell factor (R&D Systems, Minneapolis, MN; 10 ng/ml), and follicular lymphoma (Immurex, Seattle, WA; 50 ng/ml) as described previously [21, 22]. Half of the medium was changed, and the cells were split at 1:2 every 3–4 days. On day 14, floating and loosely adherent cells were harvested and used as immature DCs, which were defined by morphology, phenotype, and strong mixed lymphocyte reaction-stimulating activity. These cells were highly positive for classes I and II, CD1a, CD40, CD80, and CD86 (39–91%) and weakly positive for CD83 (15–25%) [23].

Generation of monocyte-derived DCs

Leukocyte concentrates from healthy donors were harvested after informed consent and separated into peripheral blood MNC (PBMC) by density gradient centrifugation in LSM. These cells were suspended at 1×10^6 cells/ml and cultured in six-well plates for 1 h. After being washed twice, the adherent cells were cultured with GM-CSF (500 U/ml) and IL-4 (250 U/ml) for 7 days to generate monocyte-derived DCs [24–26].

MLR in mice and humans

Splenocytes were harvested from BALB/c mice (H-2^d). T cells were purified by T cell enrichment column (R&D Systems) after lysis of contaminating erythrocytes. The purity of T cells was $\geq 90\%$. T cells (2×10^5 cells) were cultured in 96-well plates with various numbers of BM-DCs irradiated with 1500 rad from C57BL/6 mice (H-2^b). On day 3, 50 μ l culture supernatants were collected for measurement of NO and enzyme-linked immunosorbent assay (ELISA) of IFN- γ . 3 H-Thymidine (TdR; 1 μ Ci; ICN Radiochemicals, Costa Mesa, CA) was pulsed for the final 18 h, the culture was terminated, and the incorporation of 3 H-TdR was measured as previously reported [27].

Human PBMC were purified by density centrifugation with LSM. T cells were purified by a T cell enrichment column (R&D Systems) after lysis of contaminating erythrocytes (purity $\geq 90\%$) [25]. These T cells (2×10^5 cells) were cultured in 96-well plates with various numbers of allogeneic CD34⁺ cell-derived DCs irradiated with 1500 rad for 5 days. 3 H-TdR incorporation of the reactive T cells was measured as mouse MLR.

Measurement of NO

Human and mouse DCs were stimulated with IFN- γ (1000 U/ml) and/or LPS (1 μ g/ml) for 48 h. Their supernatants were harvested, and NO_x production was assessed using the Griess method [28, 29]. Briefly, aliquots of culture supernatant (50 μ l) were incubated with 50 μ l Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H₂PO₄) at room temperature (RT) for 10 min. The absorbance was measured at 550 nm in an automated plate reader. The concentration was determined with reference to a standard curve of sodium nitrite.

ELISA for mIFN- γ

The concentration of IFN- γ was measured by ELISA (PharMingen) [30]. The lower limit of detection was 15.6 pg/ml. Briefly, microtiter plates were coated overnight with anti-mouse or human IFN- γ mAb at 4°C. The plates were then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and blocked with PBS containing 10% FBS at RT for 2 h. After washing, the standards and samples were added in the wells and incubated at RT for 4 h. The wells were then washed and incubated with the biotinylated second Ab at

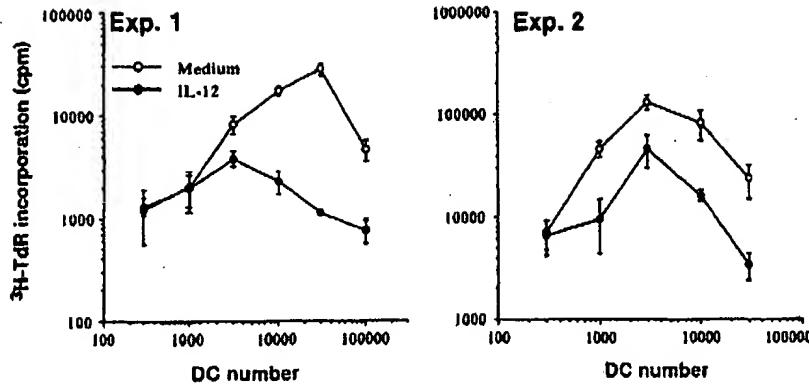


Fig. 1. The suppressive effect of IL-12 on allogeneic MLR in mice. T cells (2×10^6 cells/well) were cultured with the indicated number of irradiated (1500 rad) DCs for 4 days. The rIL-12 (1 ng/ml) was added at the initiation of culture. ^{3}H -TdR was pulsed for the final 18 h. Data are presented as mean \pm SD of triplicate culture.

RT for 45 min. Finally, the avidine-peroxidase and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) substrate were added in the well, and the absorbance at 490 nm was determined with an ELISA plate reader (Titertek Multiscan, Meckenheim, Germany).

Generation of alloreactive CTL

Mouse alloreactive CTLs were generated as described previously [31]. Briefly, splenocytes were harvested, and T cells were purified as described above. T cells (2×10^6 cells) from BALB/c mice (H-2^b) were added in 24-well plates to irradiated (1500 rad) DCs (6.7×10^4 cells) from C57BL/6 mice (H-2^b; T cell:DC ratio=30). After 5 days culture, T cells were harvested, and their lytic activity was assessed using a standard 4 h- ^{51}Cr release assay against EL-4 (H-2^a). The cytotoxicity of these alloreactive CTLs against P815 (H-2^b) was reproducibly less than 5%.

Human alloreactive CTLs were generated from peripheral blood lymphocyte-derived T cells. T cells (2×10^6 cells) purified as described above were cultured with allogeneic irradiated (1500 rad) DCs (6.7×10^4 cells) generated from CD34⁺ cells for 5 days. The cytotoxicity of these stimulated T cells was assessed for the same allogeneic DCs used as stimulators. These stimulated T cells did not show any cytotoxicity against autologous DCs generated from PBL with GM-CSF and IL-4.

The cytotoxicity of alloreactive CTL

The cytotoxicity was determined using a standard 4 h- ^{51}Cr release assay as described previously [26, 27]. In brief, 10^6 of each of the target cells was labeled with 100 μCi $\text{Na}_2^{51}\text{CrO}_4$ for 1 h. After washing twice, these effector and target cells were plated at an appropriate effector:target ratio in 96-well, round-bottom plates. The supernatant (100 μl) was collected after 4 h of incubation, and the radioactivity was counted with a γ -counter. The percentage of the specific lysis was calculated using the following formula: % specific lysis = $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$.

Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student's *t*-test. The difference was considered significant when the *P* value was less than 0.05.

RESULTS

Exogenous IL-12 suppressed the primary allogeneic MLR mediated by DCs in mice but not in humans

First, we tested the effect of IL-12 on the allogeneic MLR in mice and humans. There are no detectable IL-12 p70 heterodimers in mouse and human MLR in our system (data not shown), indicating that IL-12 is not mainly involved in alloreactive CTL generation in mouse MLR as described previously [32, 33]. It is interesting that the addition of exogenous IL-12 (1 ng/ml) suppressed MLR mediated by DCs in mice (Fig. 1). The dose dependence of IL-12-mediated suppression of MLR was confirmed in Figure 2A. IL-12 of 1 pg/ml or more was effective in inhibiting MLR. These suppressive effects were clearly found when a high number of DCs (≥ 3000 cells/well) were used for MLR as a stimulator and when IL-12 was added at the initiation of MLR (data not shown). However, when human DCs and T cells were used for MLR, IL-12 rather enhanced alloreactive T cell responses, consistent with previous reports [34] (Fig. 2B). As it has been clearly demonstrated that IL-12 is one of the growth factors of T cells [1, 2], these suppressive effects of mouse MLR by IL-12 seem to be a result of the indirect effect of IL-12 through other factors induced in MLR. IL-12 is known to stimulate IFN- γ production in T and NK cells [1, 2]. Therefore, we examined the production of IFN- γ in MLR as parallel experiments. As compared with the different effects of IL-12 on alloreactive T cell proliferation in mice and humans, IL-12 equally stimulated IFN- γ production in mouse and human MLR (Fig. 3), although the high number of DCs mediated the slight suppression of IFN- γ production in mice. These results suggested that the second factor induced

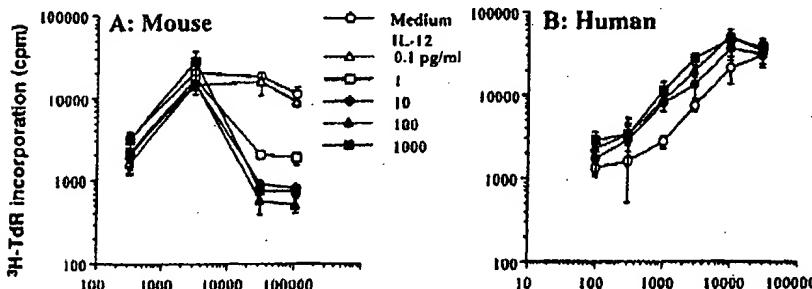


Fig. 2. Addition of IL-12 inhibits the generation of allogeneic MLR in mice but not in humans. Murine (A) and human (B) T cells (2×10^6 cells/well) were cultured with the indicated number of irradiated (1500 rad) DCs for 4 days. The various doses of IL-12 were added at the initiation of culture. ^{3}H -TdR was pulsed for the final 18 h. Data in MLR are presented as mean \pm SD of triplicate culture. Similar results were obtained in three separate experiments.

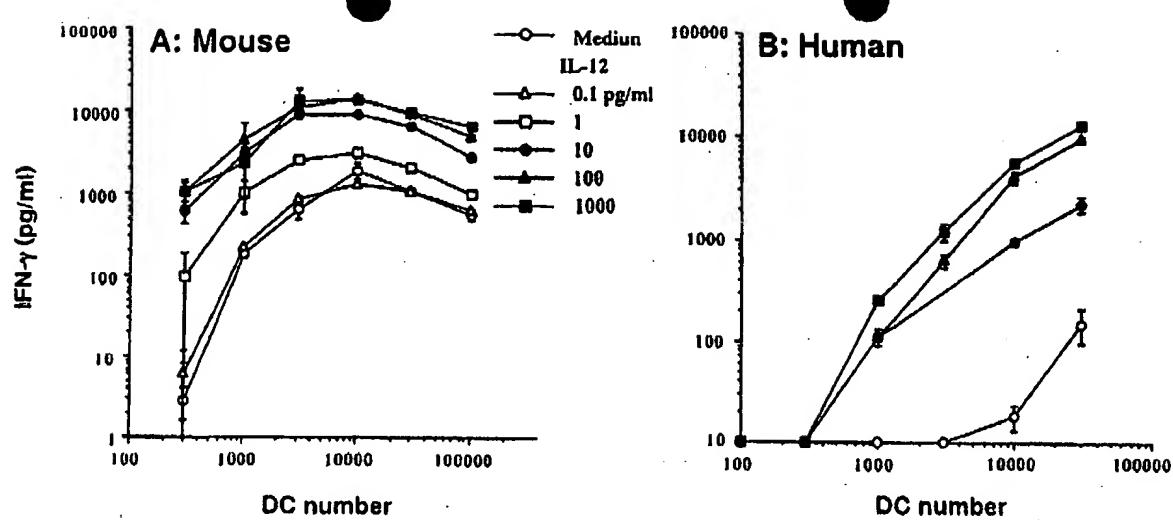


Fig. 3. IFN- γ production in murine and human MLR stimulated by DCs and IL-12. Murine (A) and human (B) T cells (2×10^5 cells/well) were cultured with the indicated number of irradiated (1500 rads) DCs for 4 days. The various doses of IL-12 were added at the initiation of culture. The supernatant (50 μ l) was harvested before the addition of ^{3}H -TdR and assessed for IFN- γ production with ELISA as described in Materials and Methods. Data are presented as mean \pm SD of triplicate culture. Similar results were obtained in three separate experiments.

by IFN- γ might be responsible for the suppressive effects of IL-12 on mouse MLR.

Addition of IFN- γ suppressed the allogeneic MLR by DCs in mice, and L-NMMA completely reversed the suppressive effects of IFN- γ

To clarify the mechanisms involved in IL-12-mediated suppression of MLR in mice, we next tested the effect of an addition of IFN- γ on MLR by DCs in mice. It is interesting that the addition of IFN- γ also suppressed the allogeneic MLR in mice (Fig. 4A). These effects were reproducibly observed when more than 10 U/ml IFN- γ was used. A recent report by Bonham et al. [15] showed that murine DCs, irrespective of their maturation stage, could produce NO in response to IFN- γ and/or LPS as well as in the allogeneic MLR. In this report, NO was shown to be the mediator for DC apoptosis. However, previous reports refer to the important role of NO as the

inhibitor of T cell proliferation [35–38]. To examine whether NO played an important role in IFN- γ -mediated suppression of mouse MLR, we tested the effect of an addition of L-NMMA, an inhibitor of NO synthase (NOS). As shown in Figure 4B, L-NMMA (0.5 mM) completely reversed the suppressive effect of IFN- γ on MLR.

L-NMMA completely abrogated the suppressive effects of IL-12 on murine allogeneic MLR

Next, the effect of L-NMMA on the IL-12-mediated suppression of mouse MLR was examined. An addition of L-NMMA enhanced allogeneic MLR stimulated with a high number of DCs in mice, as previously reported [15] (Fig. 5). Furthermore, the addition of L-NMMA completely abrogated the IL-12-mediated suppression of mouse MLR (Fig. 5). IFN- γ production, which was induced by the addition of IL-12, was further enhanced by L-NMMA in mouse MLR. In contrast, L-NMMA

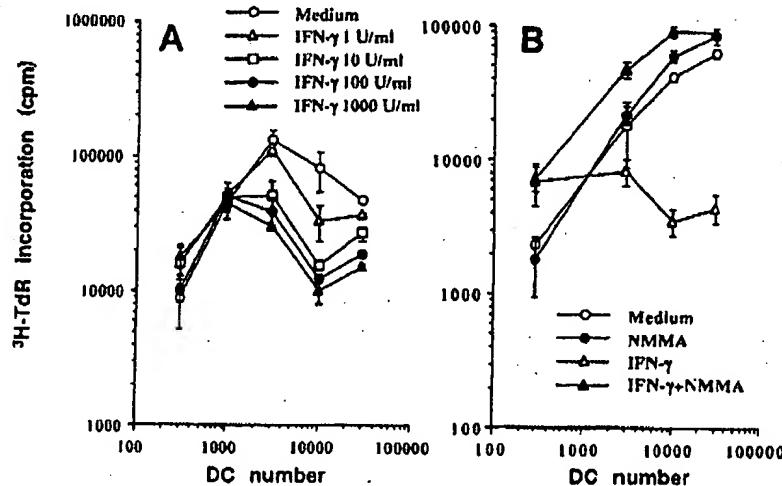


Fig. 4. Addition of IFN- γ inhibits the induction of allogeneic MLR in mice, and L-NMMA abrogates the IFN- γ -mediated suppression of MLR. T cells (2×10^5 cells/well) were cultured with the indicated number of irradiated DCs for 4 days. The various doses of IFN- γ were added at the initiation of culture (A). L-NMMA (0.5 mM) was also added in MLR in the presence of 1000 U/ml IFN- γ (B). Data in MLR are presented as mean \pm SD of triplicate culture. Similar results were obtained in two separate experiments.

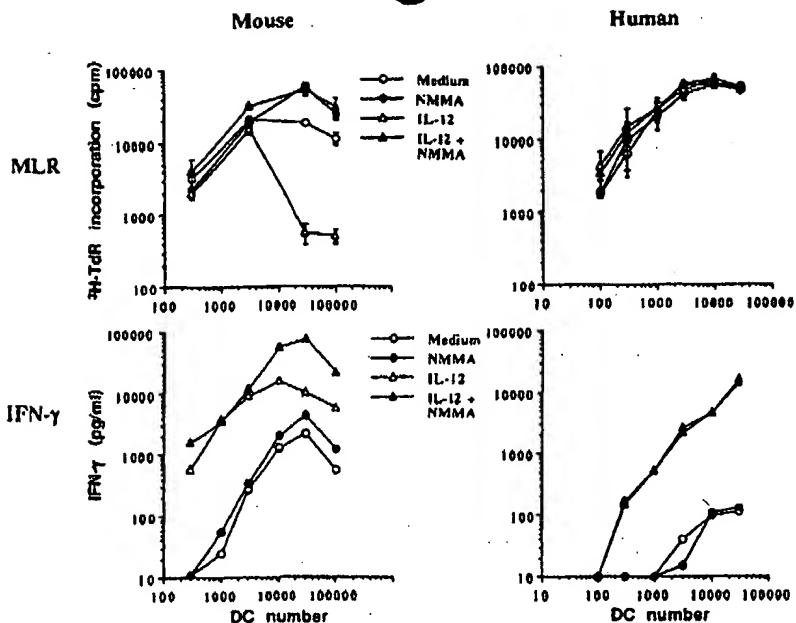


Fig. 5. L-NMMA abrogates the IL-12-mediated suppression of mouse allogeneic MLR, but human allogeneic MLR was not affected by the addition of L-NMMA. T cells (2×10^5 cells/well) were cultured with the indicated number of irradiated DCs for 4 days. L-NMMA (0.5 mM) was added to the allogeneic MLR of mice and humans. IL-12 (1 ng/ml) was used in these experiments. IFN- γ production in MLR was also assessed with ELISA. Data in MLR are presented as mean \pm SD of triplicate culture. Similar results were obtained in three separate experiments.

did not produce any effect on the proliferation and IFN- γ production in human MLR (Fig. 5).

Murine BM-DCs but not human DCs can produce a significant amount of NO in response to various stimuli

The data presented above, together with previous reports, suggested that murine DCs produce NO in MLR, which inhibits the growth of alloreactive T cells. We compared the production of NO by murine and human DCs. Figure 6 shows that a significant level of NO is detectable in mouse MLR but not in human MLR, and the addition of L-NMMA completely

suppressed NO production in mouse MLR. Furthermore, IL-12 markedly enhanced NO production in mouse MLR, whereas no detectable level of NO was found, even in the existence of exogenous IL-12 in human MLR. Next, we tested which cell population can produce NO in MLR. Table 1 shows that murine BM-DCs produce a significant amount of NO in response to IFN- γ and/or LPS, but human CD34 $^{+}$ cell- and monocyte-derived DCs do not. In this experiment, T cells from neither mice nor humans produced a detectable level of NO (data not shown). These results suggest that NO produced by DCs might play an important role in the immune response in the mouse system, especially in the presence of IL-12.

Effect of SNAP on murine and human MLR

To rule out the possibility that human T cells were insensitive for NO, we examined the effect of addition of NO in human

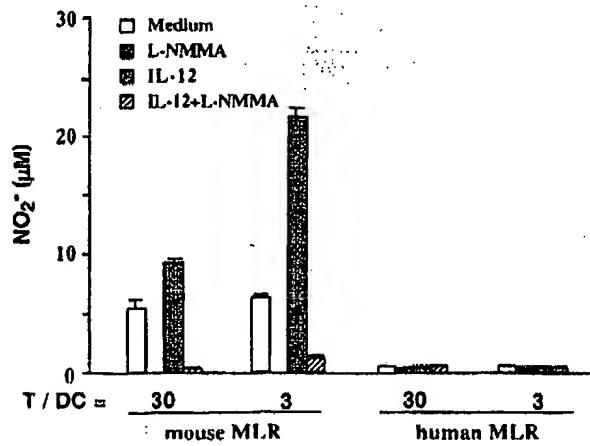


Fig. 6. NO $_2^-$ was detected in mouse MLR with DCs but not human MLR, and the addition of IL-12 significantly enhanced NO production in mouse MLR. T cells (2×10^5 cells/well) were cultured with the irradiated (1500 rad) DCs at the indicated T cell:DC ratio (T/DC) for 4 days. Their supernatant was harvested to measure the NO production in MLR using the Griess method as described in Materials and Methods. Similar results were obtained in two separate experiments.

TABLE 1. Murine BM-DCs but Not Human DCs Produce NO in Response to Various Stimuli^a

Treatment	NO $_2^-$ (μ M)		
	Murine BM-DC	CD34 $^{+}$ -DC	mo-DC
Exp. 1			
Medium	<1.0	<1.0	<1.0
IFN- γ	1.33 ± 0.16^b	<1.0	<1.0
LPS	1.87 ± 0.07	<1.0	<1.0
LPS + IFN- γ	26.03 ± 0.76	<1.0	<1.0
Exp. 2			
Medium	<1.0	<1.0	<1.0
IFN- γ	6.19 ± 1.71	<1.0	<1.0
LPS	24.22 ± 1.69	<1.0	<1.0
LPS + IFN- γ	86.85 ± 2.31	<1.0	<1.0

^a Murine and human DCs (1×10^5 cells/well) were stimulated with IFN- γ (1000 U/ml) and/or LPS (10 μ g/ml) for 48 h. The supernatant was harvested and used for measuring NO $_2^-$ with Griess reaction (sensitivity > 1.0 μ M).

^b Data are mean \pm SD of triplicate samples. mo, Monocyte.

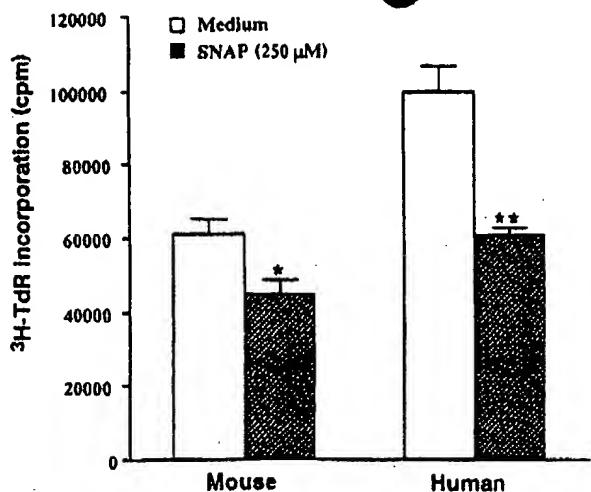


Fig. 7. Effect of addition of SNAP (NO donor) on murine and human MLR. T cells (2×10^5 cells/well) were cultured with irradiated (1500 rad) DCs (1×10^4 cells/well) for 4 days. SNAP (250 μ M) was added at the initiation of culture. ^{3}H -TdR was pulsed for the final 18 h. Data are presented as mean \pm SD of triplicate culture. *, $P < 0.01$ as compared with the group without SNAP; **, $P < 0.001$ as compared with the group without SNAP.

MLR using SNAP as a NO donor [15]. The 250 μ M SNAP was added in murine and human MLR stimulated by DCs at the initiation of culture. As shown in Figure 7, the proliferation of murine and human T cells was significantly inhibited by the treatment with SNAP, indicating that murine and human T cells were NO-sensitive.

IL-12 enhanced alloreactive CTL activity in human MLR, but L-NMMA is required to generate mouse allogeneic CTL in the presence of IL-12 in vitro

Finally, we tested the effect of IL-12 with or without L-NMMA on alloreactive CTL induction in mice and humans in vitro. As described in Materials and Methods, mouse allospecific CTL

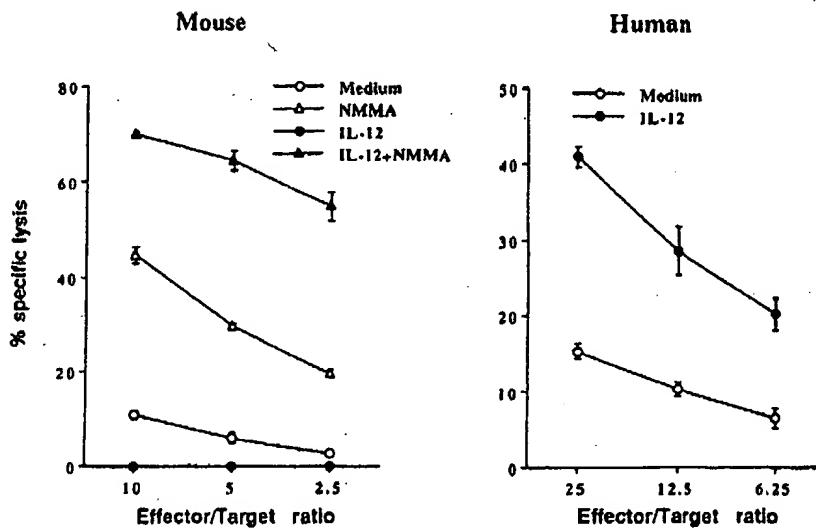
activity was determined against EL-4 (H-2^b) as compared with the control syngeneic target P815 (H-2^d). Human alloreactive CTL activity was examined against allogeneic DCs used in MLR as compared with control, autologous DCs. As previously reported [34], IL-12 (1 ng/ml) significantly enhanced alloreactive CTL activity in human MLR, whereas we could not detect any enhancing effects of IL-12 on the generation of murine alloreactive CTL in vitro (Fig. 8); rather, IL-12 strongly inhibited the allospecific CTL generation. It is interesting that the addition of L-NMMA to murine allogeneic MLR without IL-12 enhanced allospecific CTL activity. Furthermore, L-NMMA addition to murine MLR with IL-12 clearly overcame IL-12-mediated suppression of murine alloreactive CTL induction, resulting in the generation of the highest alloreactive CTL activity among all the groups tested. The CTL activity induced by the treatment of L-NMMA + IL-12 was significantly higher than that of medium + L-NMMA. In this experiment, cytotoxicity against P815 was less than 5%, indicating strict allospecificity of these CTL (data not shown).

DISCUSSION

In this report, we examined the effect of IL-12 on the primary, allogeneic MLR mediated by DCs in mice and humans. It is interesting that the addition of a high dose of rmIL-12 suppressed allogeneic MLR and CTL induction in mice but enhanced them in humans. An analysis of the mechanisms involved in the suppressive effect of IL-12 showed that NO played a critical role as an inhibitory factor for alloreactive T cell response by DCs only in mouse MLR.

IL-12 is known to play an important role in promoting Th1 differentiation and cellular immune responses. The numerous studies demonstrated the antitumor effects of IL-12, whereas the detail mechanisms remained unclear as a result of the complex effects of IL-12. The recent report by Andrews et al. [39] demonstrated that the antitumor effects of adenovirus expressing IL-12 (AdVmIL-12) were mainly mediated by the

Fig. 8. IL-12 enhanced alloreactive CTL activity in human, but L-NMMA was required for the enhancement of alloreactive CTL in mouse MLR with IL-12. Mouse T cells (2×10^6 cells) from BALB/cj mice (H-2^b) were added in 24-well plates to irradiated (1500 rad) DCs (6.7×10^4 cells) from C57BL/6 mice (H-2^b; T cell:DC ratio=30) in the presence or absence of IL-12 (1 ng/ml) with or without L-NMMA (0.5 mM). After 5 days culture, T cells were harvested for a 4 h ^{51}Cr release assay against EL-4 (H-2^b). Human alloreactive CTL were generated from PBL-derived T cells. T cells (2×10^6 cells) were cultured with allogeneic irradiated (1500 rad) DCs (6.7×10^4 cells) generated from CD34⁺ progenitor cells in the presence or absence of IL-12 (1 ng/ml) for 5 days. The cytotoxicity of these stimulated T cells was assessed for the same allogeneic DCs as the stimulators. Data are presented as mean \pm SD of triplicate culture. Similar results were obtained in two separate experiments.



inhibition of angiogenesis, as intratumoral AdVmIL-12 treatment effectively regressed the established tumor even in immune-deficient mice, including CD4- or CD8-depleted, severe combined immunodeficiency, Beige, and RAG-2-, NKT-, CD28-, and perforin-knockout mice. Furthermore, Boggio et al. [40] also reported that IL-12 prevented the spontaneous tumor development through antiangiogenic effects. Conversely, Horvath-Arcidiacono et al. [41] reported the discrepancy between the inhibitory effects of tumor growth and CTL generation in IL-12-treated mice. They found that administration of IL-12 in tumor-bearing mice eventually resulted in the rejection of tumors, but CTL activity was reduced despite the enhancement of serum IFN- γ . Noguchi et al. [9] also showed that administration of a low dose of IL-12 (1 ng/mouse) enhanced CTL activity specific for the mutated p53 peptide (234 CM) and regressed the MethA tumor expressing it, whereas a high dose of IL-12 (100 ng/mouse) suppressed 234CM-specific CTL generation. Orange et al. [10] also reported that the administration of IL-12 decreased antiviral CTL activity in the LCMV infection model. Furthermore, the report presented by Piccotti et al. [11] demonstrated that IL-12 antagonism using anti-IL-12 Ab or IL-12 p40 homodimer exacerbated cardiac allograft rejection in mice. Koblish et al. [13] recently elucidated the mechanisms involved in IL-12-mediated suppression on cellular immune responses. They reported that NO produced by adherent cells in spleen was an important factor in the IL-12-mediated immune suppression, as a NOS inhibitor completely abrogated it [13]; whereas the concentration of NO was not measured. In this study, we confirmed and extended their study using allogeneic MLR stimulated by DCs and demonstrated that murine DCs as well as macrophages were potential producers of NO. Bonham et al. [15] and Lu et al. [42] also demonstrated NO production of murine BM-DCs and that L-NMMA enhanced allogeneic MLR when a high number of DCs were used [15, 42].

These results presented above support the possibility that IL-12 has the suppressive effects on allogeneic CTL generation by enhancing NO production. It is not surprising that NO suppresses the mitogenic activity of T cells and development of allospecific CTLs, as the previous reports showed evidence that NO produced by macrophages was a suppressive factor of the proliferation of lymphocytes [43–45]. However, Piccotti et al. [46] verified the enhancement of allogeneic MLR in the presence of IL-12. Bloom and Horvath [47] reported that IL-12 did not affect allogeneic MLR. These discrepancies between the previous reports and ours might be a result of the differences of stimulator used in MLR. BM-DCs were used in our experiments and splenocytes in the other as a stimulator. Splenocytes contain a DC population, but the percentage of DCs in the spleen is 1.0–1.6% [48]. DCs are stronger stimulators in MLR when compared with splenocytes. Therefore, the stimulation by DCs might lead to a higher production of NO and the subsequent suppression of MLR. As DCs but not other APCs are known to play a key role in the induction of primary immune responses [14], experiments with DCs seem to be more important to reflect the host immune response. Our data clearly demonstrated that NO is a suppressive factor presumably produced by mouse DCs in MLR. In addition to the data of NO₂ production, the blocking experiments with L-NMMA corroborated the inhibitory effect of NO on mouse MLR. Furthermore, the treatment with L-NMMA clearly enhanced allogeneic CTL activity. These data suggest that the combined use of IL-12 and L-NMMA might be effective for treatment of tumors in mice. In fact, Koblish et al. [13] reported that inhibition of NOS function using L-NMMA enhanced IL-12-induced delay of SCK tumorigenesis. Furthermore, as Gajewski and co-workers [49] reported, the use of a low dose of IL-12 might be better in combination with DC-based immunotherapy.

In the present study, exogenous IFN- γ also suppressed allogeneic MLR, and the addition of L-NMMA abrogated IFN- γ -mediated suppression. There are many controversial reports in which IFN- γ enhances or inhibits CTL generation. The recent study using mice with the IFN- γ gene knocked out showed that allospecific CTL activity of IFN- $\gamma^{-/-}$ mice was higher than that of IFN- $\gamma^{+/+}$ mice, indicating that the existence of IFN- γ somehow inhibits the generation of allospecific CTL [50]. As IFN- γ stimulates NO production, it is likely that these results might have been affected by the condition of NO production in culture.

Conversely, exogenous human IL-12 has been reproducibly reported to enhance antigen-specific human CTL induction by DCs from CD8 $^{+}$ T cells in vitro [34, 39]. Chouaib et al. [34] showed that IL-12 was involved in human allogeneic MLR and the addition of rhIL-12-enhanced, allospecific CTL activity using a blocking experiment with anti-human IL-12 antibody. Consistent with these results, we demonstrated in this report that IL-12 (1 ng/ml) enhanced allospecific CTL activity in humans. This difference between murine and human CTL induction by IL-12 was a result of the different production of NO by DCs. The present study was the first report describing that human CD34 $^{+}$ cell- and monocyte-derived DCs did not produce a significant amount of NO in response to LPS and/or IFN- γ as well as in allogeneic MLR.

These results suggest that NO production by APCs including macrophages and DCs is an important factor regulating the effect of IL-12 in mice. The combined use of L-NMMA might reverse the suppressive effects of IL-12 on the induction of CTLs in mice and more closely mimic immune responses mediated by IL-12 in humans. As NO induced by IL-12 in vivo is likely to be less in humans, the antitumor effect mediated by IL-12 via cellular immune responses might be expected in humans rather than in mice.

ACKNOWLEDGMENTS

This work was supported by Grant PO1 CA59371 of U.S.A. (M. T. L., P. D. R., and H. T.), a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture, and a grant from the Ministry of Health and Welfare of Japan (Y. N. and S. S.). The authors thank Drs. Michael R. Shurin, Hiromune Shimamura, and Catherine Haluszczak for their help with DC culture and flow cytometry. We also thank Mrs. Susan Schoonover and Lori McKenzie for their technical assistance and Fumie Kaneko for the purification of human monocytes by an elutriator.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:

Docket No.:

Serial No.:

Group Art Unit:

Filing Date:

Examiner:

For:



DECLARATION OF SHERMAN FONG, Ph.D. UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Sherman Fong, Ph.D. declare and say as follows: -

1. I was awarded a Ph.D. in Microbiology by the University of California at Davis, CA in 1975.
2. After postdoctoral training and holding various research positions at Scripps Clinic and Research Foundation, La Jolla, CA, I joined Genentech, Inc., South San Francisco, CA in 1987. I am currently a Senior Scientist at the Department of Immunology/Discovery Research of Genentech, Inc.
3. My scientific Curriculum Vitae is attached to and forms part of this Declaration.
4. I am familiar with the Mixed Lymphocyte Reaction (MLR) assay, which has been used by me and others under my supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project.
5. The MLR assay is a well known and widely used proliferative assay of T-cell function, the basic protocols of which are described, for example, in Current Protocols in Immunology Vol. 1, Richard Coico, Series Ed., John Wiley & Sons, Inc., 1991, Unit 3.12. (Exhibit A). This publication is incorporated by reference in the description of the MLR protocol in the present application.

6. The T-lymphocytes or "T-cells" of our immune system can be induced to proliferate by a variety of agents. The MLR assay is designed to study a particularly important induction mechanism whereby responsive T-cells are cultured together (or "mixed"), with other lymphocytes that are "allogeneic", e.g. lymphocytes that are taken from different individuals of the same species. In the MLR protocol of the present application, a suspension of PBMCs that includes responder T-cells, is cultured with allogeneic PBMCs that predominantly contain dendritic cells. According to the protocol, the allogeneic "stimulator" PBMCs are irradiated at a dose of 3000 Rad. This irradiation is done in order to create a sample of cells that has mainly dendritic cells. It is known that the dendritic cell population among the PBMCs are differentially affected by irradiation. At low doses (500-1000 Rad), the proliferation of most cells, including the B cells in the PBMCs, is preserved, however, at doses above 2000 Rad, this function of B cells is abolished. Dendritic cells on the other hand, maintain their antigen presentation function even at a 3000 Rad dose of radiation. (See, e.g. Current Protocols in Immunology, supra, at 3.12.9). Accordingly, under the conditions of the MLR assay used to test the PRO polypeptides of the present invention, the stimulator PBMCs remaining after irradiation are essentially dendritic cells.
7. Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naive T cells *in vivo*. They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells. For further details about the properties and role of dendritic cells in immune-based therapies see, e.g. Steinman, Drug News Perspect. 13(10):581-586 (Exhibit B).
8. The MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

9. Such immune stimulants find important clinical applications. For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured *in vitro* and returned to the patient to stimulate anti-cancer response. [Thurner et al. J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)].
10. It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant. Some PRO polypeptides do the reverse, and give inhibition of T-cell proliferation in the MLR assay. It is my considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, as specified in the present application, would be expected to find practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases.

Dated: 6/16/04

By: Sherman Fong

Sherman Fong, Ph.D.

Sherman Fong, Ph.D.

Senior Scientist
Department of Immunology
Genentech Inc.
1 DNA Way.
South San Francisco, California 94080-4990

Work Telephone: (650) 225-2783 FAX: (650) 225-8221
Home Telephone: (510) 522-5411

Education:

1978 - 1980 Postdoctoral Fellow in Immunology, Research Institute of Scripps Clinic,
Scripps Clinic and Research Foundation, La Jolla, California

1975 - 1978 Postdoctoral Fellow in Immunology, University of California at San Francisco, San Francisco, California

1970 - 1975 Ph.D. in Microbiology, University of California at Davis, California

1966 - 1970 B.A. in Biology/Microbiology, San Francisco State University, San Francisco, California

Professional Positions:

Currently: Senior Scientist, Department of Immunology/Discovery Research, Genentech, Inc., South San Francisco, California

8/00-8/01 Acting Director, Department of Immunology, Genentech, Inc. South San Francisco, California

10/89 Senior Scientist in the Department of Immunology/Discovery Research, Genentech, Inc.
South San Francisco, California

3/89 - 10/89 Senior Scientist and Immunobiology Group Leader, Department of Pharmacological Sciences, Immunobiology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

9/87 - 3/89 Scientist, Department of Pharmacological Sciences, Immunopharmacology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

1/82 - 9/87 Assistant Member (eq. Assistant Professor level), Department of Basic and Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

6/80 - 12/81 Scientific Associate in the Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

7/78 - 6/80 Postdoctoral training in the laboratory of Dr. J. H. Vaughan, Chairman, Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

2/75 - 6/78 Postdoctoral training in the laboratory of Dr. J. W. Goodman, Department of Microbiology and Immunology, School of Medicine, University of California, San Francisco, California

7/71 - 12/74 Research Assistant and Graduate Student, Department of Medical Microbiology, School of Medicine, University of California, Davis, California, under Dr. E. Benjamini

Awards:

Recipient: National Institutes of Health Postdoctoral Fellowship Award (1975).

Recipient: Special Research Award, (New Investigator Award), National Institute of Health (1980).

Recipient: P.I., Research Grant Award, National Institute of Health (1984).

Recipient: Research Career Development Award (R01), National Institutes of Health (1985).

Recipient: P.I., Multi-Purpose Arthritis Center Research Grant, NIH (1985)

Recipient: P.I., Research Grant Award, (R01 Renewal), National Institute of Health (1987).

Scientific Associations:

Sigma Xi, University of California, Davis, California Chapter

Member, The American Association of Immunologists

Committee Service and Professional Activities:

Member of the Immunological Sciences Study Section, National Institutes of Health Research Grant Review Committee, (1988-1992).

Advisory Committee, Scientific Review Committee for Veteran's Administration High Priority Program on Aging, 1983.

Ad Hoc member of Immunological Sciences Study Section, National Institutes of Health, 1988.

Ad Hoc Reviewer: Journal of Clinical Investigations, Journal of Immunology, Arthritis and Rheumatism, International Immunology, Molecular Cell Biology, and Gastroenterology

Biotechnology Experience

Established at Genentech in 1987-1989 within the Immunobiology Laboratory, in the Department of Pharmacological Sciences, group to study the immunogenicity of recombinant hGH (Protropin®) in hGH transgenic mice.

Served as Immunologist on the Biochemical Subteam for Protropin® Project team.

Served as Immunologist on the Met-less hGH and Dnase project teams, two FDA approved biological drugs: second generation hGH Nutropin® and Pulmozyme® (DNase).

Served immunologist in 1989-1990 on the CD4-IgG project team carrying out in vitro immunopharmacological studies of the effects of CD4-IgG on the in vitro human immune responses to mitogens and antigens and on neutrophil responses in support of the filing of IND to FDA in 1990 for use of CD4-IgG in the prevention of HIV infection. Product was dropped.

In 1989-1991, initiated and carried research and development work on antibodies to CD11b and CD18 chains of the leukocyte β2 integrins. Provided preclinical scientific data to Anti-CD18 project team

supporting the advancement of humanized anti-CD18 antibody as anti-inflammatory in the acute setting. IND filed in 1996 and currently under clinical evaluation.

1993-1997, **Research Project Team leader** for small molecule $\alpha 4\beta 1$ integrin antagonist project. Leader for collaborative multidisciplinary team (N=11) composed of immunologists, molecular/cell biologists, protein engineers, pathologists, medicinal chemists, pharmacologists, pharmaceutical chemists, and clinical scientists targeting immune-mediated chronic inflammatory diseases. Responsible for research project plans and execution of strategy to identify lead molecules, assessment of biological activities, preclinical evaluation in experimental animals, and identification of potential clinical targets. Responsible for identification, hiring, and working with outside scientific consultants for project. Helped establish and responsible for maintaining current research collaboration with Roche-Nutley. Project transferred to Roche-Nutley.

1998-present, worked with Business Development to identify and create joint development opportunity with LeukoSite (currently Millennium) for monoclonal antibody against $\alpha 4\beta 7$ intergrin (LDP-02) for therapeutic treatment for inflammatory bowel disease (UC and Crohn's disease). Currently, working as scientific advisor to the core team for phase II clinical trials for LDP-02.

Currently, **Research Project Team Biology Leader** (1996-present) for small molecule antagonists for $\alpha 4\beta 7$ /MAdCAM-1 targeting the treatment of human inflammatory bowel diseases and diseases of the gastrointestinal tract. Responsible for leading collaborative team (N=12) from Departments of Immunology, Pathology, Analytical Technology, Antibody Technology, and Bio-Organic Chemistry to identify and evaluate lead drug candidates for the treatment of gastrointestinal inflammatory diseases.

Served for nearly fifteen years as **Ad Hoc reveiwer** on Genentech Internal Research Review Committee, Product Development Review Committee, and Pharmacological Sciences Review Committee.

Worked as **Scientific advisor** with staff of the **Business Development** Office on numerous occasions at Genentech, Inc. to evaluate the science of potential in-licensing of novel technologies and products.

2000-2001 Served as Research Discovery representative on Genentech Therapeutic Area Teams (Immunology/Endocrine, Pulmonary/Respiratory Disease Task Force)

Invited Symposium Lectures:

Session Chairperson and speaker, American Aging Association 12th Annual National Meeting, San Francisco, California, 1982.

Invited Lecturer, International Symposium, Mediators of Immune Regulation and Immunotherapy, University of Western Ontario, London, Ontario, Canada, 1985.

Invited Lecturer, workshop on Human IgG Subclasses, Rheumatoid Factors, and Complement. American Association of Clinical Chemistry, San Francisco, California, 1987.

Plenary Lecturer, First International Waaler Conference on Rheumatoid Factors, Bergen, Norway, 1987.

Invited Lecturer, Course in Immunorheumatology at the Universite aux Marseilles, Marseilles, France, 1988.

Plenary Lecturer, 5th Mediterranean Congress of Rheumatology, Istanbul, Turkey, 1988.

Invited Lecturer, Second Annual meeting of the Society of Chinese Bioscientist of America, University of California, Berkeley, California, 1988.

Lecturer at the inaugural meeting of the Immunology by the Bay sponsored by The Bay Area Bioscience Center. The β 2 Integrins in Acute Inflammation, July 14, 1992.

Lecturer, "Research and Development -- An Anatomy of a Biotechnology Company", University of California, Berkeley, Extension Course, given twice a year--March 9, 1995 to June 24, 1997.

Lecturer, "The Drug Development Process - Biologic Research - Genomics", University of California, Berkeley Extension, April 21, 1999, October, 1999, April 2000, October, 2000.

Lecturer, "The Drug Development Process - Future Trends/Impact of Pharmacogenomics", University of California Berkeley Extension, April 2001, October 2001, April 2002.

Invited Speaker, "Targeting of Lymphocyte Integrin $\alpha 4\beta 7$ Attenuates Inflammatory Bowel Diseases", in Symposium on "Nutrient effects on Gene Expression" at the Institute of Food Technology Symposium, June, 2002.

Patents:

Dennis A. Carson, Sherman Fong, Pojen P. Chen.

U.S. Patent Number 5,068,177: Anti-idiotype Antibodies induced by Synthetic Polypeptides, Nov. 26, 1991

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim and Steven R. Leong.....

U.S. Patent Number 5,677,426: Anti-IL-8 Antibody Fragments, Oct. 14, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent Number 5,686,070: Methods for Treating Bacterial Pneumonia, Nov. 11, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent 5,702,946: Anti-IL-8 Monoclonal Antibodies for the Treatment of Inflammatory Disorders, Dec. 30, 1997

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong.

U.S. Patent Number 5,707,622: Methods for Treating Ulcerative Colitis, Jan. 13, 1998

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,074,873: Nucleic acids encoding NL-3, June 13, 2000

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,348,351 B1: The Receptor Tyrosine Kinase Ligand Homologues. February 19, 2002

Patent Applications:

Sherman Fong, Kenneth Hillan, Toni Klassen

U.S. Patent Application: "Diagnosis and Treatment of Hepatic Disorders"

Sherman Fong, Audrey Goddard, Austin Gurney, Daniel Tumas, William Wood

U.S. Patent Application: Compositions and Methods for the Treatment of Immune Related Diseases.

Sherman Fong, Mary Gerritsen, Audrey Goddard, Austin Gurney, Kenneth Hillan, Mickey Williams, William Wood . U.S. Patent Application: Promotion or Inhibition of Cardiovasculogenesis and Angiogenesis

Avi Ashkenazi, Sherman Fong, Audrey Goddard, Austin Gurney, Mary Napier, Daniel Tumas, William Wood. US Patent Application: Compounds, Compositions and Methods for the Treatment of Diseases Characterized by A33-Related Antigens

Chen, Filvaroff, Fong, Goddard, Godowski, Grimaldi, Gurney, Hillan, Tumas, Vandlen, Van Lookeren, Watanabe, Williams, Wood, Yansura

US Patent Application: IL-17 Homologous Polypeptides and Therapeutic Uses Thereof

Ashkenazi, Botstein, Desnoyers, Eaton, Ferrara, Filvaroff, Fong, Gao, Gerber, Gerritsen, Goddard, Godowski, Grimaldi, Gurney, Hillan, Kljavin, Mather, Pan, Paoni, Roy, Stewart, Tumas, Williams, Wood US Patent Application: Secreted And Transmembrane Polypeptides And Nucleic Acids Encoding The Same

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Library of Congress Cataloging in Publication Data:

Current protocols in immunology / edited by John E. Coligan ... [et al.]

p. cm.

Kept up to date by quarterly supplements.

4 vols. Includes index.

ISBN 0-471-52276-7

1. Immunology—Laboratory manuals I. Coligan, John E.

[DLM: 1. Clinical Protocols. 2. Immunologic Techniques. QW 525 C976]

QR183.C87 1991

616.07'0'072—dc20

DNLM/DLC

for Library of Congress

90-15726

CIP

Printed in the United States of America

Proliferative Assays for T Cell Function

UNIT 3.12

A number of agents can specifically or nonspecifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes—in contrast to helper function for B lymphocytes (*UNIT 3.10*) or cytotoxicity (*UNIT 3.11*)—proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immunocompetence of an animal. In addition, the assays described in this unit form the basis for identifying the appropriate cellular population that might be used to obtain T cell clones (*UNIT 3.13*) or T cell hybridomas (*UNIT 3.14*).

The assays have been divided into two groups on the basis of whether they are used to stimulate primed or unprimed T lymphocytes. The first basic protocol describes the use of agents that are capable of activating unprimed T lymphocytes in culture either by pharmacologic means (calcium ionophore and phorbol ester stimulation), by direct cross-linking of the T cell receptor (TCR) on a large percentage of responder cells (anti-CD3, anti-TCR- $\gamma\delta$, or anti-TCR- $\alpha\beta$ monoclonal antibodies), by cross-linking the receptors on certain subpopulations of T cells with monoclonal antibodies specific for the V regions of β chains of the TCR (anti-V β) or with enterotoxins specific for certain V β -chain regions, or by indirectly cross-linking the TCR (lectins or monoclonal antibodies to non-TCR antigens). The first alternate protocol describes the use of plate-bound antibodies specific for the TCR to stimulate proliferation. The second alternate protocol describes the activation of unprimed T cells to cell-associated antigens in the mixed leukocyte reaction (MLR). The first support protocol describes the preparation and use of T cell-depleted accessory or stimulator cells and the second support protocol describes methods for blocking accessory cell proliferation. Finally, the second basic protocol describes the induction of a T cell proliferative response to soluble protein antigens or to cell-associated antigens against which the animal has been primed *in vivo*.

The assays in this unit employ murine T lymphocytes. Induction of proliferative responses of murine B lymphocytes is described in *UNIT 3.10*. Related assays for use with human peripheral blood lymphocytes are described in *UNIT 7.9*.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

ACTIVATION OF UNPRIMED T CELLS

Unprimed T cells can be induced to proliferate by a variety of agents, including pharmacological agents, anti-CD3/TCR or anti-Thy-1 monoclonal antibodies, enterotoxins and lectins. The commentary briefly describes the specificities of these agents, while Table 3.12.1 lists sources and concentrations for use in this protocol. Although this procedure is intended to measure proliferation of T cells specifically, in many cases induction of T cell proliferation is dependent on the presence of non-T cells that function as accessory cells. The latter provide additional costimulatory signals for T cell proliferation as well as cross-link (via their Fc receptors) monoclonal antibodies bound to cell-surface antigens. The requirement for non-T accessory cells varies with the nature of the stimulatory ligand and can range from absolute dependence to accessory cell-independent T cell activation (see Table 3.12.1). The activation is calculated after determining the difference in incorporation of [3 H]thymidine between stimulated and control cells.

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Table 3.12.1 Agents Used to Activate Unprimed T Cells in Proliferative Assays

Agent ^a	Source/ cat. no. ^b	Concentration	Accessory cells ^c	Mode of action, etc.
PMA	SIG P8139	1-10 ng/ml	No	Use with ionomycin or A23187; pharmacologic
Ionomycin	CAL 407950	200-500 ng/ml	No	Use with PMA; pharmacologic
A23187	CAL 100105	100-500 ng/ml	No	Use with PMA; pharmacologic
PHA	WD HA16	1-5 µg/ml	Yes	Indirect TCR cross-linking
Con A	PH 17-0450-01	1-10 µg/ml	Yes	Indirect TCR cross-linking
Anti-Thy-1	PG mAb-G7	1-50 µg/ml	Yes ^c	Indirect TCR cross-linking
Anti-CD3	PG HM-CD3	0.1-5 µg/ml	Yes ^c	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-αβ	PG HM-AB-TCR	0.1-10 µg/ml	Yes ^c	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-γδ	PG HM-GD-TCR-1; HM-GD-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-8.1, 8.2 ^c	PG MM-Vβ-TCR-1	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-6 ^c	PG RM-Vβ-TCR-2	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-11	PG RM-Vβ-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Staph tox A	TT AT101	1-10 µg/ml	Yes ^c	Vβ-1,3,10,11,17-receptor specificity
Staph tox B	TT BT202; SIG S4881	1-100 µg/ml	Yes ^c	Vβ-3,7,8,17-receptor specificity
Staph tox E	TT ET404	1-10 µg/ml	Yes ^c	Vβ-11,15,17-receptor specificity

^aAbbreviations: PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; Con A, concanavalin A; Staph tox A, B, & E, *Staphylococcus* enterotoxins A, B, & E.

^bSupplier addresses and phone numbers are provided in APPENDIX 5. Abbreviations: CAL, Calbiochem; PG, Pharmingen; PH, Pharmacia LKB; SIG, Sigma; TT, Toxin Technology; WD, Wellcome Diagnostics.

^cWhen using anti-CD3 and anti-TCR antibodies in soluble form (rather than plate-bound), accessory cells are required. When using Staph enterotoxins, accessory cells must express appropriate MHC class II molecules. Accessory cell dependence is not absolute with anti-Thy-1 antibodies.

Materials

Complete RPMI-5 and RPMI-10 media (*APPENDIX 2*)

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNIT 3.1*)

Activating agent(s) (Table 3.12.1)

Phosphate-buffered saline (PBS; *APPENDIX 2*)

Accessory cells: unfractionated mouse spleen cell suspension, irradiated or treated with mitomycin C (second support protocol) or T cell-depleted (first support protocol)

[³H]thymidine (*APPENDIX 3*)

15- and 4-ml disposable, polystyrene conical tubes with screw caps

Low-speed centrifuge with Sorvall H-1000B rotor (or equivalent)

1-, 5-, and 10-ml disposable polystyrene pipets

96-well flat- or round-bottom microtiter plates with lids (Costar #3596 or #3799)

25- to 100- μ l single- and multichannel pipettors with disposable tips

Additional reagents and equipment for removing organs (*UNIT 1.9*), preparing single-cell suspensions (*UNIT 3.1*), and counting, labeling, and harvesting cells (*APPENDIX 3*)

1. Prepare responder leukocyte suspensions from thymus, spleen, or lymph node in complete RPMI-5 as described in *UNIT 3.1*.

The size of the intended experiment dictates the number of organs to be collected. See annotation to step 3 for an indication of cell number required, and UNIT 3.1 for number of cells per organ. Spleen, thymus, and lymph node can be used as responder cells, while only spleen is a source of accessory cells. Purified T cells or subpopulations of T cells (i.e., CD4⁺ or CD8⁺) cells may also be used. See UNITS 3.1-3.6 for enrichment/depletion methods.

2. Centrifuge single-cell suspensions in 15-ml conical tubes for 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 \times g), room temperature, and discard supernatant.
3. Resuspend cell pellet in complete RPMI-5. Count responder cells and adjust to $\sim 10^6$ cells/ml with complete RPMI-10.

While this concentration (1 \times 10⁶ cells/ml or 2 \times 10⁵ cells/well) will give satisfactory responses with most cell populations, it is useful to compare 2, 4, and 8 \times 10⁵ cells per well in initial pilot experiments. If unfractionated spleen or lymph node cells are used as the responder population, sufficient accessory cells are present and there is no need to supplement the cultures with additional cells. However, if highly purified T cells or T cell subpopulations are used as responders, it will be necessary to add non-T accessory cells depending on the nature of the activating agent (see Table 3.12.1). This is most easily accomplished by adding increasing numbers (0.1, 0.5, and 1.0 \times 10⁵) of syngeneic spleen (accessory) cells in 0.1 ml to 2 \times 10⁵ T cells in 0.1 ml (see first support protocol). Also, a meaningful comparison of the responsiveness of different cell populations requires titrations of both the activating agents as well as the responding cell populations, and a kinetic experiment.

4. Prepare working solutions of activating agents in 4-ml conical tubes at room temperature as follows. For MAb, toxin, or lectin, make a series of four dilutions from 1 mg/ml stock solutions—e.g., 100, 30, 10, and 3 μ g/ml in PBS. For the pharmacological agent, make single dilutions of 100 ng/ml solution of PMA and 1 μ g/ml A23187 (or 4 μ g/ml ionomycin) in PBS.

If MAb in supernatant or ascites form are being used, at least four dilutions should also be used. Working solutions should be used immediately, since the various proteins, especially MAb, may bind to the plastic.

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See Table 3.12.1 for V β specificities of staphylococcal enterotoxins. It is essential to verify that the mouse strain employed expresses the MHC class II surface molecules for which the enterotoxin has a specific binding affinity. See Marrack and Kappler (1989) for further discussion of various enterotoxins and their specificities.

5. Add 20 μ l of each dilution of activating reagent (MAb, enterotoxin or lectin) to each of three wells of a 96-well flat- or round-bottom microtiter plate. Include control wells with 20 μ l of PBS only. Add 20 μ l PMA or calcium ionophore at the single concentration indicated in step 4, as the dose-response curve for these agents is extremely narrow.

A series of four dilutions will form one row of each microtiter plate, allowing for efficient organization of the plates.

6. To the wells of the 96-well microtiter plate containing activating agent, add 2×10^5 cells in 0.2 ml.
7. Place microtiter plates in a humidified 37°C, 5% CO₂ incubator for 2 to 4 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions and must be determined empirically (see critical parameters).

8. Add [³H]thymidine to each well. Return the plates to CO₂ incubator to pulse 18 to 24 hr. Harvest cells using a semiautomated sample harvester and measure cpm in β scintillation counter.
- 9a. Compute the data as the difference in cpm of stimulated (experimental) and control (no activating agent added) cultures. This is done by subtracting the arithmetic mean of cpm from triplicate control cultures from the arithmetic mean of cpm from corresponding stimulated cultures. The results are referred to as " Δ cpm."
- 9b. Alternatively, compute the data as the ratio of cpm of stimulated and control cultures. This is done by dividing the arithmetic mean of cpm from stimulated cultures by the arithmetic mean of cpm from control cultures. The results are referred to as "SI" (stimulation index).

The second method (step 9b) has the disadvantage that small changes in background values will result in large changes in SI and should be interpreted with caution. In most publications, Δ cpm rather than SI values are preferred.

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ACTIVATION OF UNPRIMED T CELLS WITH PLATE-BOUND ANTIBODIES

Although it is possible to induce T cell activation with monoclonal antibodies to the CD3/TCR complex in solution during culture, such activation depends on cross-linking of the antibody by Fc receptor-bearing accessory cells. This protocol describes the use of monoclonal antibodies to the CD3/TCR complex by coupling them to the wells of the microtiter plates. The T cell proliferative response induced under these conditions does not require the presence of significant numbers of accessory cells, although the responses obtained may be suboptimal (Jenkins et al., 1990).

Use of this protocol is recommended for use with those antibodies to the CD3/TCR complex which bind poorly to the Fc receptor present on murine accessory cells and which do not induce T cell activation in soluble form. Although all monoclonal antibodies readily couple to plastic under these conditions, it is very difficult to induce a proliferative response with certain antibodies such as the G7, anti-Thy-1 monoclonal antibody. In such cases, the conditions described in the basic protocol should be followed.

Additional Materials

PBS (APPENDIX 2), room temperature and 4°C

1 mg/ml purified anti-CD3 or anti-TCR MAb in PBS (for nonspecific activation of T cells) or 1 mg/ml purified anti-V β or anti-TCR- $\gamma\delta$ MAb in PBS (for activation of T cells with specific receptors; see Table 3.12.1)

1. In 4-ml conical polystyrene tubes, prepare a series of four dilutions of MAb from sterile 1 mg/ml stock solutions—e.g., 100, 10, 1, and 0.1 μ g/ml—using room temperature PBS.

Sources and recommended concentrations of monoclonal antibodies can be found in Table 3.12.1; since MAb will bind to plastic, the working dilutions should be used immediately.

The ability of anti-TCR antibodies to cross-link receptor molecules varies depending on the purity of the MAb preparation and the affinity of the MAb for the TCR/CD3 complex. Optimum dilutions will have to be determined in dose-response experiments. Alternatively, preparations of ascites fluid from the MAb can be tested at different dilutions (e.g., 1:100, 1:200, 1:400, and 1:800), but use of purified antibody will allow for better standardization of the assay.

Because the efficacy of MAb-induced activation depends on the amount of antibody bound to the bottom of the wells, it is crucial to make the dilutions in a buffer without any additional source of proteins such as FCS or albumin; these would compete with the binding of the antibody, and therefore reduce the responsiveness. For this reason, it is also not recommended to perform the assay with culture supernatants of the appropriate hybridomas.

2. Add 30 μ l of each concentration of MAb solution to each of three wells of a 96-well round-bottom microtiter plate. Include control wells of 30 μ l PBS only.

A series of four dilutions will form one row of each plate, allowing for efficient organization of the plates. Consistently better responses are seen with round-bottom (compared with flat-bottom) plates in antibody-mediated experiments.

Most often, optimal responses are seen with 10 μ g/ml antibody. There is no point in adding more than the indicated amount of antibody, since the maximum amount that can bind to surface of the wells is ~2 to 3 μ g (A.M.K., unpub. observ.).

3. Cover the plate and gently tap its side to ensure complete covering of the bottom of the wells. Incubate plates 90 min at 37°C. During incubation, proceed to step 4.

During this incubation, the antibodies bind to the plastic in the wells for subsequent cross-linking of the T cell receptors on responding T cells. Plates can also be prepared the night before an experiment and kept in the refrigerator overnight, after the 37°C incubation.

4. Prepare responder cell suspensions as in steps 1 to 3 of the basic protocol.

Highly purified T cell populations can be used in these studies as the proliferative response induced is accessory cell-independent. However, the presence of non-T accessory cells does not interfere with the proliferative response.

5. Wash the wells of the incubated plates by adding 200 μ l cold PBS and inverting the plates with a flick of the hand on a stack of paper towels placed in a tissue culture hood. Repeat washing procedure two more times to remove excess antibody.

6. To the wells of the washed plates, add $\sim 2 \times 10^5$ cells in 0.2 ml.

If cells are not ready at this stage, plates may be kept in the refrigerator overnight after 100 μ l PBS has been added. Presumably, longer storage periods should be acceptable, but our experience is limited to ≤ 4 day periods. The PBS should be removed before the cells are added.

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Most cell populations will give peak responsiveness at this cell dosage, but pilot experiments should be performed to establish optimal conditions.

7. Proceed as in steps 7 to 9 of the basic protocol, but incubate cultures for 2 to 3 days before adding [³H]thymidine.

Kinetic assays should be performed to determine the optimum culture period.

T CELL PROLIFERATION IN MIXED LYMPHOCYTE CULTURES

In the mixed lymphocyte culture (MLC) or reaction (MLR), suspensions of responder T cells are cultured with allogeneic stimulator lymphocytes. The activating stimulus is the foreign histocompatibility antigen (usually MHC class I or class II molecules) expressed on the allogeneic stimulator cells. Responder cells need not be primed because a sufficiently high number of T cells in the MLC will respond to the stimulator population. If the stimulator cell population contains T cells, their uptake of [³H]thymidine must be prevented by irradiation or treatment with mitomycin C; alternatively the stimulator cell suspension can be depleted of T cells (see support protocols).

Additional Materials

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNITS 1.9 & 3.1*) or purified T cells or T cell subpopulations (*UNITS 3.1-3.6*)

Stimulator cells: allogeneic mouse spleen cells that differ from the responder cells at *H-2* or *Mls* loci, irradiated or treated with mitomycin C (second support protocol) or T cell-depleted (first support protocol)

1. Prepare responder cell populations as in steps 1 to 3 of the basic protocol. Although unfractionated cell populations can be used as responders in certain situations, it may be preferable to use purified T cells or T cell subsets.

To estimate the MLR of a cell population, it is necessary to perform a dose-response assay with different numbers of responder cells. Typically, three replicate wells are set up containing each of the following: 0.5, 1, 2, and 4 × 10⁵ cells (optimal responses are usually obtained with the latter two densities). The setup for these four cell densities will occupy one row (12 wells) of a microtiter plate.

For thymocytes, it may be necessary to use 8 × 10⁵ cells per well because the frequency of responding T cells is lower; the lowest number of responder cells could then be 1 × 10⁵ and the doses in between would be 2 and 4 × 10⁵. Using this range of higher numbers of responder cells may also be preferred when experimental manipulations are expected to reduce the frequency of responding T cells.

2. To a 96-well microtiter plate, add 5 × 10⁴ to 4 × 10⁵ responder cells in 0.1 ml to each well. For each experimental group, set up three replicate wells.

*Stimulation of leukocytes for proliferation in 96-well microtiter plates can be run in parallel with cytotoxic T lymphocyte (CTL) generation (*UNIT 3.11*), which is performed in 24-well microtiter plates. For example, cells can be diluted to 4 × 10⁶ cells/ml and added to 24-well plates in 1.0 ml/well for CTL generation and to 96-well plates in 0.1 ml/well for proliferation.*

3. Prepare a single-cell suspension of irradiated or mitomycin C-treated stimulator cells. Alternatively, prepare a suspension of T-cell depleted stimulator cells. Add 0.1 ml to each well of the plates containing responder cells.

The optimum number of stimulator cells must be determined for each MLC and for different responder cells. For a range of responder cells from 0.5-4 × 10⁵, test stimulator cells at densities of 2, 4, and 8 × 10⁵/ml (i.e., 2, 4, and 8 × 10⁵/well). It should be noted that the stimulator cell suspension provides both the specific antigen to be recognized by the responder T cells as well as nonspecific accessory cells. If

highly purified T cells are used as the responder population, it is therefore not necessary to supplement the cultures with non-T accessory cells syngeneic to the responder T cells.

Separate wells with control cultures should be set up that include—for each dose of responder and stimulator cells—replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells. Values obtained from these controls reflect “background” proliferation values (see step 9 of basic protocol). Other negative controls often included are wells with stimulator cells alone and wells with responder cells alone. These are not used for the calculation of the data, but are useful to compare with the background proliferation values; the latter should not be much higher (<2-fold) than those obtained with stimulator or responder cells alone. Higher background values indicate potential autoreactivity.

4. Follow steps 7 to 9 of the basic protocol, but incubate the cultures for 3 to 6 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions, and must be determined empirically (see critical parameters).

DEPLETION OF T CELLS FROM ANTIGEN-PRESENTING/STIMULATOR CELL SUSPENSIONS

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Although normal unfractionated spleen cell populations can be used as a source of accessory cells, in certain types of experiments it may be preferable to use spleen cell populations from which the T cells have been removed. This procedure ensures that none of the observed proliferative responses of the responder population result from T cell factors derived from the accessory cell population. For example, even T cells whose cell division has been blocked (second support protocol) can produce cytokines. In the following steps, T cell-depleted spleen cell suspensions are prepared using a lytic monoclonal antibody to the T cell antigen, Thy-1. Because almost all the antigen presentation or stimulator cell activity in spleen resides in the non-T cell fraction, this procedure also leads to enrichment of functional antigen-presenting cell function. Further enrichment of antigen-presenting cells (APC) by flotation of the T cell-depleted spleen cells on Percoll gradients is also described. Other procedures leading to enrichment of APC are described elsewhere; the method described in *UNIT 3.7* does not deplete T cells and therefore is not recommended here; the method described in *UNIT 3.15* leads to higher levels of enrichment that are not required in the protocols presented here.

Additional Materials

Spleen cells from nonimmunized mice
Hanks balanced salt solution (HBSS; *APPENDIX 2*)
Low-Tox rabbit complement (Cedarlane #CL3051), reconstituted with ice-cold distilled water and filter-sterilized
Anti-Thy-1.2 ascites (HO-13-4; ATCC #TIB 99) or anti-Thy-1.1 ascites (HO-22-1; ATCC #TIB 100; alternatively, see Table 3.4.1 for other anti-Thy-1 MAb and *UNIT 2.6* for production of ascites)
70% Percoll solution (*UNIT 3.8* and reagents and solutions)

1. Centrifuge the spleen cell suspension derived from single spleen down to a pellet.

The spleen cells should always be from nonprimed animals and should be syngeneic to the responder T cells unless they are to be used as stimulator cells in the MLC.

2. To the pellet, add 0.9 ml HBSS, 0.1 ml complement, and 25 µl anti-Thy-1 ascites.

If cells from more than a single spleen are needed, the procedure should be scaled up accordingly.

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The choice of anti-Thy-1 reagent to be used depends on the strain of animal from which the spleen was derived. The great majority of commonly available mouse strains (except AKR) express the Thy-1.2 allele.

3. Incubate the mixture at 45 min in a 37°C water bath.
4. Centrifuge 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 × g), room temperature, and discard supernatant. Resuspend pellet in HBSS and wash two more times.
5. Count viable cells (APPENDIX 3) and resuspend in complete RPMI-10 or PBS for inactivation as in the second support protocol, or in HBSS to prepare low-density accessory cells (see below).

The T cell-depleted spleen cell population is comprised of B cells, macrophages, and dendritic cells. Further enrichment of cells with enhanced accessory cell function can be obtained by fractionation of this population on Percoll.

6. Dilute 70% Percoll solution to 55% by mixing 23.58 ml of the 70% Percoll with 6.42 ml HBSS. Resuspend T cell-depleted spleen cells from step 5 in HBSS at 20×10^6 cells/ml.
7. Layer 3 ml cell suspension over 3 ml of 55% Percoll solution in a 15-ml conical centrifuge tube.
8. Spin 13 min in H-1000B rotor at 3000 rpm (1900 × g), room temperature.
9. Remove cells that band at the Percoll/HBSS interface with a 5-in. Pasteur pipet and wash 3 times in HBSS as in step 4.
10. Count viable cells and resuspend in complete RPMI-10 for inactivation according to the second support protocol.

The population obtained from steps 6 to 10 is comprised of large cells including macrophages, dendritic cells, and activated B lymphocytes. This population of cells is enriched in accessory cell function. When used in either of the basic protocols with purified T responder cells, fewer of the Percoll-purified cells should be needed to provide accessory function.

SUPPORT PROTOCOL

BLOCKING CELLULAR DIVISION OF ACCESSORY/STIMULATOR CELLS

There are two situations in which inhibition of accessory or stimulator cell division should be blocked. When purified T cells rather than unfractionated lymphoid populations are used in the basic protocol, cultures are frequently supplemented with accessory cells syngeneic to the responder T cells. If accessory cell DNA synthesis is inhibited, one can then be certain that the resultant proliferative response is comprised entirely of responder T cells and does not contain a component of recruited B cell proliferation derived from the accessory cell populations. In the MLR, the stimulator cells are spleen cells from mice that differ from the responder cells in *H-2* and/or *Mls* gene expression (see APPENDIX 1, Tables A.1C.1 and A.1F.1) and they can also recognize alloantigens on the responder cells. This responsiveness of stimulator cells against responder cells in an MLR (so-called back-stimulation) must be prevented by blocking cellular division. This can be done by treatment of stimulator cells with mitomycin C (a DNA cross-linking reagent) or by g irradiation. Many investigators prefer mitomycin C treatment when antigenic differences encoded for by *Mls* genes are to be measured, or when an irradiation source is not available. For more information on the loci encoding *Mls* genes, see Tables A.1F.2 and A.1F.3.

Mitomycin C Treatment

Additional Materials

Mitomycin C (Sigma #M-0503; store in dark)

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1. In a 15-ml aluminum foil-wrapped tube, prepare a solution of mitomycin C in PBS at 0.5 mg/ml and filter sterilize.

Since mitomycin C is very light-sensitive, it is necessary to prepare a fresh stock solution each day for each experiment.
2. Prepare spleen cell suspension as described in steps 1 and 2 of the basic protocol at a concentration of 5×10^7 cells/ml in PBS.
3. Add mitomycin C to a final concentration of 50 $\mu\text{g}/\text{ml}$ (100 $\mu\text{l}/\text{ml}$ of cell suspension) and wrap the tube in aluminum foil. Incubate 20 min at 37°C.
4. Add an excess of complete RPMI-5 (i.e., fill tube with ~12 ml) and centrifuge 10 min in Sorvall H-1000B rotor at 1200 rpm (300 $\times g$). Discard supernatant and repeat washing procedure two more times.

Three washes are crucial, because any traces of mitomycin C left among the cells will reduce proliferative responses when the cells are added to an MLC.
5. Resuspend pellet in complete RPMI-10. Count cells with hemacytometer. Adjust to desired concentration as described in the annotation to step 6 of the basic protocol.

Irradiation Treatment

Prepare a spleen cell suspension as described in steps 1 to 3 of the basic protocol, at a final concentration of $5-10 \times 10^6$ cells/ml in complete RPMI-10. Using a source of ionizing irradiation (^{60}Co or ^{137}Cs γ -irradiator; e.g., Gammacell 1000, Nordion), deliver 1000 to 2000 rad of irradiation to the cells.

This dose range of irradiation is suitable for most immunologic applications employing spleen cell suspensions. However, antigen presentation by different spleen cells is differentially affected by irradiation (Ashwell et al., 1984): at low doses (500 to 1000 rad), antigen-presenting function of B cells is preserved; after doses of 1100 to 2000 rad, a substantial decline is observed; and doses >2000 rad abolish the participation of B cells as APC. Macrophages and dendritic cells, on the other hand, maintain antigen presentation through doses of 3000 rad. To ensure that B cells do not participate in the responses measured, some investigators prefer to use doses of 2000 rad. However, responsiveness to *Mls* antigens can best be measured with stimulator cells that received doses of <1000 rad, since B cells present *Mls* more effectively. Alternatively, *Mls* responsiveness can be measured after mitomycin C treatment of stimulator cells, since it also preserves the antigen-presentation function of B cells.

When transformed cell lines are used as antigen-presenting or accessory cells, higher doses must be used to ensure blockage of cell division. The appropriate dose will have to be determined empirically for each cell line, but is likely to be at least 5000 rad; some transformed cell lines require as much as 10,000 to 12,000 rad, and may be more sensitive to mitomycin C treatment.

ACTIVATION OF PRIMED T CELLS

Proliferative responses to viruses, protein antigens, minor transplantation antigens, and the male H-Y antigen require *in vivo* immunization followed by *in vitro* stimulation. Furthermore, enhanced proliferative responses to those antigens that will generate primary *in vitro* responses (i.e., MHC antigens) can be obtained by *in vivo* priming. Multiple immunizations usually elevate *in vitro* responses.

To immunize animals for *in vitro* secondary responses to soluble protein antigens or peptides, dissolve antigens and emulsify in complete Freunds adjuvant (UNIT 2.5). For strong responses by draining lymph node cells, immunize animals in a hind footpad. For

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strong responses by spleen cells, immunize intraperitoneally. Tail-base immunization also can be used as an efficient route of immunization; follow procedure for intradermal injection. To prime animals against cellular antigens, inject intraperitoneally with $1-5 \times 10^7$ cells that express the antigen. Immunization protocols are described in *UNIT 1.6*.

Within 2 to 3 weeks after in vivo priming, in vitro responsiveness of primed T cells can usually be measured. This assay is often used as a preparation for subsequent in vitro cloning procedures (*UNIT 3.14*) and T cell hybridoma preparation (*UNIT 3.13*).

Materials

Complete RPMI-10 medium (*APPENDIX 2*)

Responder cells: Purified T cells isolated from lymph nodes (*UNITS 3.1-3.6*) of in vivo primed mice

Antigen: 1 mg/ml sterile protein antigen(s) (*UNIT 3.13*), in PBS or suspension of irradiated or mitomycin C-treated stimulator cells expressing alloantigens at 8×10^6 cells/ml (*UNIT 3.11*, support protocol) in complete RPMI-10 medium (*APPENDIX 2*)

Accessory cells: suspension of irradiated or mitomycin C-treated (or T cell-depleted) spleen cells syngeneic to the responding T cells at 5×10^6 cells/ml in complete RPMI-10 medium

4-ml conical tubes

96-well flat-bottom microtiter plates with lids

1. Follow steps 1 to 3 of the first basic protocol for preparation of responder cells.
2. Prepare 4-fold dilution series of the antigens in 4-ml conical tubes, using complete RPMI-10.

The following dilutions are recommended: 100, 10, 1, and 0.1 µg/ml protein antigens and 8, 4, 2, and 1×10^6 cells/ml of stimulator cells in complete medium.

3. Add antigens to 96-well flat-bottom microtiter plates, at 30 µl/well for protein antigens or 100 µl/well for cellular antigens. For each experimental group, set up three replicate wells and include control wells with medium only (no antigen).

By using four concentrations of antigens and three replicate wells for each dose, one row of a microtiter plate will cover the entire tested range.

4. Add responder T cells in 0.1 ml to each well.

Purified T cells are recommended; otherwise extremely high background values may be obtained. This appears to be due in part to proliferation of recruited cells (T and non-T) that are not antigen-specific. If unfractionated lymph node cells from recently primed mice are used, add $1-2 \times 10^5$ cells per well and proceed to step 6.

5. If purified lymph node T cells specific for protein antigens are used, add 0.1 ml of accessory spleen cells syngeneic to the donor of the responder T cells at 5×10^6 cells per well.

Purified T cells require an exogenous source of accessory non-T cells. Accessory cells function both as antigen-presenting cells and as a source of undefined "second signals." They are not required for cell preparations primed against cellular antigens, because accessory cell function is provided by the stimulator cells.

6. Proceed as in steps 7 to 9 of the basic protocol.

Culture periods before labeling can vary widely and kinetic assays should be performed. In general, for T cells from primed mice, it is likely that the response will peak at day 4 or 5.

REAGENTS AND SOLUTIONS

Percoll solution

Diluent:

45 ml 10× PBS, pH 7.4 (APPENDIX 2)

3 ml 0.6 M HCl

132 ml H₂O

Filter sterilize

70% Percoll solution:

63 ml Percoll (Pharmacia LKB #170891-01)

37 ml sterile diluent (above)

Final osmolarity should be 310 to 320 osM

COMMENTARY

Background Information

Proliferative assays for measuring T cell function have certain advantages and disadvantages compared to the cytotoxic T lymphocyte (CTL) assay described in UNIT 3.11 or the lymphokine production assays in UNITS 3.15 & 6.3. Advantages are that proliferative assays are less time-consuming, less labor-intensive, less cell-consuming, and less expensive than "true" effector T cell function assays. A disadvantage is that antigen specificity is not as easily demonstrated in proliferative assays as in CTL assays, unless antigen-specific clones of proliferating cells are used. Furthermore, the proliferative assay only detects dividing cells instead of measuring true effector T cell function.

It is not clear which T cell function is measured in proliferative assays; the proliferative response should therefore be used solely as general indicators of T cell reactivity. Data obtained in proliferative assays might variously reflect proliferation of CTL, lymphokine-producing T cells, or nonactivated "by-stander" cells, and will be severely affected by the function of non-T cells such as accessory cells (see below). Since the majority of T cells respond to and produce IL-2 upon activation, differences in responsiveness in a proliferative assay in part reflect differences in IL-2 production by the responding T cells. Proliferative assays therefore become more meaningful when combined with the lymphokine detection assays presented in UNITS 3.15 & 6.3. Since responsiveness to IL-2 is also determined by the levels and functionality of IL-2 receptors, further information will be added by including measurements of IL-2 receptors (UNIT 6.1) or by flow cytometry (UNIT 5.4). Yet, as a first approximation of cellular activation, proliferative assays are valuable.

Critical Parameters and Troubleshooting

Parameters affecting the magnitude of T cell proliferative responses include cell concentration, type of medium, source of serum, incubator conditions (CO₂ level and humidity), type and concentration of activating agent, type of responding T cells, type of accessory/stimulator cells, mouse strain, and culture time. Optimal conditions for individual laboratories and experiments must be derived empirically with respect to these variables, but general guidelines are provided below.

A number of agents can be employed in the first basic protocol to induce T cell proliferation (Table 3.12.1). T cells may be activated by pharmacologic means by producing an elevation of intracellular free calcium with a calcium ionophore combined with activation of protein kinase C with a phorbol ester. The most direct means of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the CD3/TCR complex—i.e., anti-CD3, anti-TCR- $\alpha\beta$ or $\gamma\delta$, as well as anti-V β antibodies that are capable of interacting with a subset of cells bearing a specific TCR. A vigorous T cell proliferative response of defined subsets can also be induced with certain bacterial toxins known as staphylococcal enterotoxins. These toxins are often referred to as "superantigens" (Marrack and Kappler, 1989) because they stimulate T cells via the variable (V) gene segment of the TCR. Different toxins have affinities for different V β chains and these specificities make them valuable reagents for activating T cells. The activating capacity of toxins is also dependent on their ability to bind to MHC class II molecules (i.e., responding T cells react with the toxin/class II complex); thus, responsiveness varies with the

In Vitro Assays
for Mouse B and
T Cell Function

3.12.11

mouse strain used. Lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) have been widely used for many years to activate T cells. Although the precise mechanism of action of these agents is unknown, it is likely that lectins activate T cells by indirectly cross-linking the TCR because TCR-negative cells will not respond to these agents. Lastly, it is also possible to induce T cell activation with monoclonal antibodies to cell-surface antigens other than the TCR; this protocol employs the G7 monoclonal antibody, one of the most effective of the anti-Thy-1 activators (Gunter et al., 1984).

When comparing the reactivity of different cell populations, it is essential to perform dose-response assays for responder T cells and activating agents and for both responder and stimulator T cells (in MLR), since each population may yield optimal responses at different cell numbers. This may reflect differences in frequency of responding cells, and hence may indicate a need to perform limiting dilution assays (*UNIT 3.15*). Since peak responsiveness of different populations of T cells may occur at different times, it is also essential to perform kinetic experiments—i.e., compare responsiveness at days 2, 3, 4, and 5.

Differences in responsiveness need not necessarily be due to differences in the frequency of responding T cells, but may also indicate differences in the efficacy with which co-stimulatory activity or “second signals” are delivered by the accessory cells present in different cell populations. The type of interactions pertinent to the generation of primary responses by T cells is explained in the commentaries of *UNITS 3.8, 3.11, & 3.13*. Specific requirements for inducing activation with immobilized antibodies have been described (Staerz and Bevan, 1986; Hathcock et al., 1989; Jenkins et al., 1990). A responding cell population completely devoid of accessory cells (such as purified populations of splenic or lymph node T cells or cloned T cells) will yield fine responsiveness in an MLC, since accessory cell function is provided by the stimulator cells; however, the same population will generally not yield responses when mitogens, antigens, or enterotoxins are used. In such a setting, accessory cells may also function as antigen-presenting cells (APC). Addition of irradiated or mitomycin C-treated syngeneic sources of accessory cells (either whole spleen cells or purified APC; see first support protocol) can be used to restore responsiveness in purified T cells. The need for accessory cells can sometimes be

bypassed when anti-TCR monoclonal antibodies are coupled to plastic, or when certain anti-Thy-1 monoclonal antibodies are used; however, these conditions do not necessarily result in optimal responsiveness (Jenkins et al., 1990).

The level of [³H]thymidine incorporation should not be regarded only as a reflection of cellular proliferation: some nondividing cells will synthesize DNA and “cold” thymidine released by disintegrating cells will compete with incorporation of labeled thymidine. Therefore, measurements of DNA synthesis should be accompanied by counting viable cells over the length of the culture period if a true estimate of cellular proliferation is to be obtained. Of course, cell death of nonactivated cells will also interfere with the accuracy of this last parameter.

The sensitivity of proliferation assays is such that small errors in cell numbers will result in large differences in [³H]thymidine incorporation values. When values obtained in triplicate cultures correspond poorly (e.g., >5% difference in cpm values >1000), technical problems such as cell clumping, dilution, and pipetting should be considered. Excessively high values may be obtained from contaminated wells, as [³H]thymidine will be incorporated into replicating bacteria; therefore, it is good practice to check the wells from microtiter plates under an inverted microscope for contamination. Contamination may also interfere with proliferation of the activated lymphocytes.

It is also useful to check for blast formation by microscopic examination of the cultures: activated lymphocytes will tend to enlarge, and detection of blasts will give a general indication of successful activation.

The main problem that may occur with proliferative response assays is high levels of background [³H]thymidine incorporation in control cultures without antigens. This problem is frequently due to the fetal calf serum (FCS) used to supplement the cultures, which may be mitogenic for B cells. Different lots of FCS should be screened to select those that are nonstimulatory or only weakly stimulatory in the absence of other stimuli, and that support strong proliferative responses upon antigenic stimulation of T cells.

If flat-bottom microtiter plates are used in the procedure and weak responses occur, it may be useful to switch to round-bottom plates. Our laboratory has found consistently better responses in round-bottom plates when

thymocytes are used as responder cells or with slight alloantigenic differences between responding and stimulating cells. In addition, antibody-mediated experiments yield better results with round-bottom plates. Presumably, this reflects better cell contact obtained in such plates; optimal responses will almost certainly occur at different cell numbers than in flat-bottom plates and densities will have to be adjusted accordingly.

Although satisfactory responses to most alloantigens can be obtained with complete RPMI-10 medium, it may be necessary to compare different media. This need arises when the proliferative responses are weak (i.e., when [³H]thymidine values for activated cultures are <10-fold higher than those for control cultures) and may occur under various circumstances: weak alloantigenic differences between responder and stimulator cells, weak T cell proliferative function in the responder cells or diminished APC function in the stimulator cells due to experimental manipulations, or a low precursor frequency of responding T cells. Thymocytes in particular do not contain a high level of responding T cells. Frequently, proliferation can be improved when complete Clicks or Dulbeccos media are used (with additives as described in APPENDIX 2), presumably because these media contain additional nutrients and have an osmolarity more compatible with mouse serum than RPMI.

When RPMI is used as medium, 5% CO₂ will be sufficient, but for other media, a 7.5% CO₂ concentration in the incubator will be more satisfactory. Generally, the buffering capacity of DMEM is insufficient at 5%, but fine at 7.5%. Much will also depend on the proliferative activity of the responding population of T cells (e.g., vigorous proliferation will reduce the pH in the cultures); it is therefore recommended to compare responsiveness in initial pilot experiments in incubators set at different CO₂ concentrations.

The culture period required for stimulation—after which the cells are to be labeled—varies for different laboratories, media, and types of responding and stimulator cells. Conditions eliciting weak responses, such as those obtained with thymocytes or a weak alloantigenic difference, will require a longer culture period (5 to 6 days) than those which elicit a higher frequency of responding T cells (3 to 4 days). Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time for T cell prolifer-

ation. Addition of [³H] thymidine on days 2, 3, 4, 5, and 6 will provide a useful test; further extension of the culture period will not yield any improvements, due to exhaustion of nutrients in the medium.

Anticipated Results

For proliferative assays described in the basic protocol, which activate the majority of the responding T cells, responses of 100,000 cpm should be obtained; in the MLR or following activation with monoclonal antibodies to subpopulations of T cells (anti-V β), responses up to 100,000 cpm may be observed; however, measurements of 20,000 cpm (with tight standard errors) can be quite satisfactory. Background values of <1000 cpm should be expected. Reported results (as described in step 9a) should be mean cpm of experimental wells minus background cpm (Δ cpm).

Time Considerations

The time required to set up proliferative assays is not more than a day, with the number of hours depending on the number of different groups of responder cells that must be prepared. The time required for incubation of cells ranges from 2 to 6 days, as noted above in critical parameters. Following an additional 18- to 24-hr incubation period for pulsing, harvesting the cells and measuring cpm will require several hours depending on the number of plates (~15 min for harvesting each plate and ~100 min for counting each plate at 1 min/sample).

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Contributed by Ada M. Kruisbeek
Netherlands Cancer Institute
Amsterdam, The Netherlands

Ethan Shevach
National Institute of Allergy and
Infectious Diseases
Bethesda, Maryland

LOOKING AHEAD

Dendritic cells are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

The Dendritic Cell Advantage: New Focus For Immune-Based Therapies

by Ralph M. Steinman

The focus of immune therapeutics has been on lymphocytes, the cellular mediators of immunity, and the suppression of lymphocyte function. The drug cyclosporin (cyclosporine) is an excellent and successful example. However, medicine needs therapies that enhance immunity or resistance to infections and tumors. Medicine also needs strategies, whether suppressive or enhancing, that are specific to the disease-causing stimulus or antigen. In contrast to lymphocytes, dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. DCs capture antigens and then initiate and control the activities of lymphocytes, including the development of resistance to infections and tumors (reviewed in references 1-3).

Summary

Dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. The best-studied function of DCs is to convert antigens into immunogens for T cells. The "DC advantage" entails a myriad of functions. DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity. Another special feature of DCs is their location and movement in the body; DCs are stationed at surfaces where antigens gain access to the body. The events that make up the life history of DCs are now being unraveled in molecular terms. As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent. © 2000 Prous Science. All rights reserved.

The controlling role of DCs is best known for thymus-dependent lymphocytes or T cells which are important in many diseases, the most poignant being the AIDS epidemic (Table 1). DCs were identified in a few laboratories that were focusing on the induction of immunity from resting T cells. It was noted that immune tissues (spleen, lymph nodes, lymph, blood) had a small fraction of cells with unusual

"tree-like" or "dendritic" processes. These distinctive cells had not been recognized previously and they proved to have distinct functions. Most importantly, DCs were potent inducers of immunity even in animals, not just the test tube, and now even in patients (reviewed in references 1-3).

The DC field was held back by the fact that there were so few cells relative

TABLE I: HUMAN DISEASES THAT INVOLVE T CELLS

- Rejection of organ transplants and graft-vs.-host disease in bone marrow transplantation
- Resistance to many infections including vaccine design
- Vaccines against tumors and immune therapies for existing tumors
- Allergy
- AIDS
- Autoimmune diseases like insulin-dependent (juvenile) diabetes, multiple sclerosis, rheumatoid arthritis and psoriasis

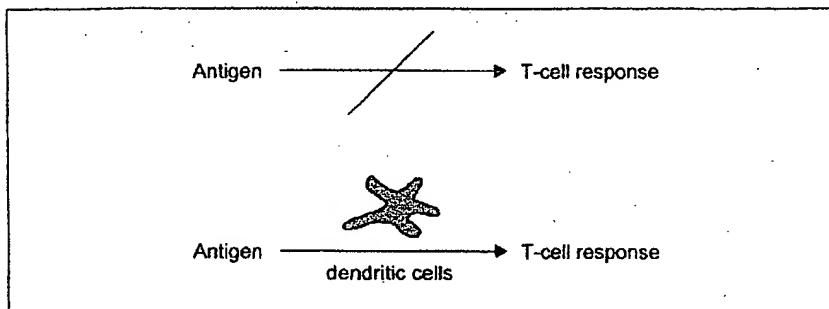


Fig. 1. A key function of dendritic cells. Antigens within tumors, transplants and infectious agents need to be presented by DCs to become immunogens, i.e., to make T cells begin to grow and exhibit their helper and killer functions.

to other players in the immune system such as B cells, T cells and macrophages. In reality, DCs are quite abundant for the job they have to do, namely, to initiate immune responses from antigen-specific T cells. In immune system organs like lymph nodes, DCs form an extensive network throughout the T cell-rich regions and physically outnumber any given antigen-reactive T cell by at least 100 to 1. The DC field was also held back because many thought that the cells were no different from macrophages, thus keeping investigators from working on the active DCs. In reality, DCs were identified on the basis of profound differences from macrophages, and their many distinct properties and functions were only uncovered by separating DCs from macrophages.

The best-studied function of DCs is to convert antigens into immunogens for T cells. The antigen receptors on T cells do not focus on intact proteins in microbes and tumors, but instead recognize fragmented or processed proteins, that is, peptides. The processing of protein antigens into peptides occurs within cells, and then the peptides are

displayed or presented at the cell surface affixed to products of the major histocompatibility complex (MHC). The ensuing interaction between a T-cell receptor (TCR) and its specific MHC-peptide complex allows a T cell to detect peptides formed within cells in transplants, tumors, sites of infection and self tissues attacked during autoimmune disease (Table I). "Antigens" refers to specific substances recognized by the immune system, while "immunogens" refers to antigens that effectively induce responses either by themselves or together with enhancing materials called "adjuvants." For T cells in particular, antigens and immunogens are not one and the same (Fig. 1). Even preprocessed peptides and MHC-peptide complexes are weak immunogens. This was evident early on in the work of Peter Medawar, the great scientist who discovered the immune basis of transplantation. He spent many years trying to purify functioning transplantation antigens. These efforts were to little avail.

What was not known in Medawar's time is that transplantation antigens

(later shown to be MHC-peptide complexes) become immunogenic when presented by DCs.⁴ In other words, transplantation antigens when presented on many cell types are weak immunogens, but on DCs they become powerful inducers of immunity.⁴ The same is true of peptides that become much more immunogenic when presented on DCs. DCs activate T cells by getting them to divide and express their helper and killer functions. Then the activated T cells interact with other antigen-presenting cells to eliminate the antigen in question. DCs are also called "nature's adjuvant," because prior adjuvants were artificial substances used to enhance immunity. The DC advantage entails a myriad of functions, some of which will be considered below.

Potency of dendritic cells in initiating immunity in tissue culture

What are some specific features of DCs that warrant attention? The first is their potency. Very small numbers of DCs are sufficient to trigger strong T-cell responses in test tubes. Immune assays are generally carried out with impure antigen-presenting cells, applied at a dose of one presenting cell for every T cell, the latter often preactivated. In contrast, roughly one DC per 30–100 T cells is more than sufficient to induce optimal responses, including responses by resting T cells. A single DC can simultaneously activate 10–20 T cells nestled within its sheet-like processes. Therefore, DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity.

It has always been clear that the accessory function of DCs did not depend exclusively on their capacity to process antigens to form MHC-peptide complexes. This is because the stimuli that were used to define the potency and immune-activating role of DCs did not require that the DCs process an applied antigen. Such stimuli included major transplantation antigens, mitogens, contact allergens, anti-

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T-cell antibodies and superantigens. Furthermore, once resting T cells were activated by DCs, the T cells responded vigorously to antigens presented by other cell types, showing that the latter were not deficient in forming ligands for the antigen receptor on T cells, but instead lacked accessory properties.

The word "accessory" has since been replaced by the terms "professional" and "co-stimulatory," but the basic concept is unchanged by shifting terminology. T cells need stimuli other than their specific trigger or ligand (MHC-peptide complexes) to begin to grow and function, for example, to produce the interleukins and killer molecules mentioned above. DCs are potent in providing the needed accessory or co-stimulatory functions. For example, DCs produce an adhesion molecule called DC-SIGN that binds to a target on resting T cells called ICAM-3,⁵ and DCs express very high levels of a stimulatory molecule called CD86 that binds to CD28 on resting T cells.⁶ These are but two examples of the specialized activities of DCs. These cells do not operate as a single magic bullet.

Position of dendritic cells *in vivo*

Another special feature of DCs is their location and movement in the body. As criteria were developed to identify DCs, it became feasible to go back into the animal and patient to look for the corresponding cells in different tissues. DCs are stationed at surfaces where antigens gain access to the body (Fig. 2, left). The skin and the airway have been the best studied. DCs are found in afferent lymphatic vessels, special channels that allow cells to move from peripheral tissues to lymphoid organs, primarily the T-cell areas (Fig. 2, middle and right). This migration is most readily observed in models of skin transplantation and contact allergy, which are the two most powerful immune responses known.

DC migration is likely to be very important. The body's pool of T cells primarily traffics through the T-cell areas of lymph nodes, rather than

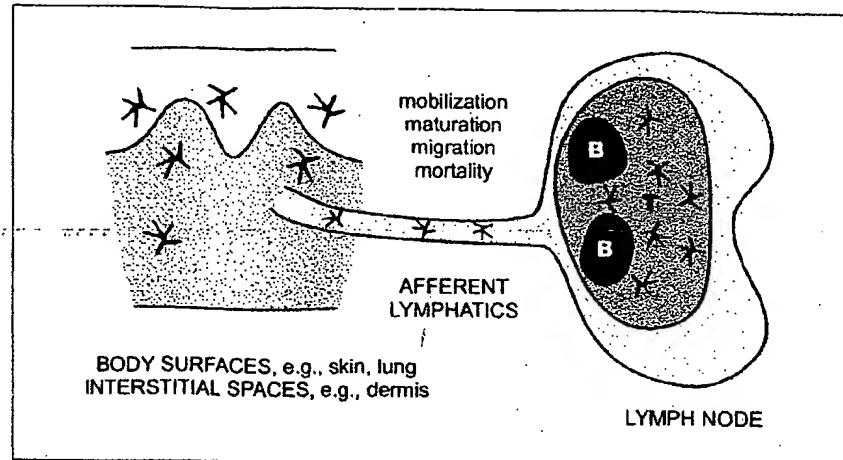


Fig. 2. Distribution of dendritic cells *in situ*. DCs at body surfaces and in solid organs can pick up antigens, move to the lymphoid tissues to find antigen-specific T cells and initiate immunity. Molecular mechanisms are being uncovered that govern the mobilization, maturation, migration and mortality of these DCs. In the lymph node, T lymphocytes are selected for expansion and differentiation into helper and killer T cells. The activated T cells then leave the lymph node to return to the body surface or peripheral organ to eliminate the antigen.

through tissues where antigens are usually deposited. So when DCs capture antigens in the skin, airway or another peripheral tissue, their migration to the T-cell areas gives them a chance to select the corresponding rare specific T cells from the assembled repertoire (Fig. 2). The selected T cells then increase in numbers (clonal expansion) and function, enabling the specific immune response to begin. The initial frequency of T cells that recognize an antigen is very small. Only one in 10,000–100,000 of T cells in the repertoire responds to a specific MHC-peptide complex. Therefore, it is so precise and efficient for DCs to be able to pick up an antigen in the periphery and then initiate the immune response from rare T-cell clones in lymphoid organs.

The events that make up the life history of DCs (Figs. 2 and 3) are now being unraveled in molecular terms. For example, scientists are figuring out how to expand antigen-capturing precursors to DCs using IL3 ligand and granulocyte colony-stimulating factor (G-CSF). Key players for the mobilization of DCs from the periphery to lymph nodes are the multidrug resistance receptors, usually studied for their capacity to mediate resistance to chemotherapeutic agents rather than

movement of DCs. Migration of DCs is controlled by chemokines produced in the lymphatic vessels and lymphoid organs (Fig. 2). These act on DC chemokine receptors to orchestrate their movement to the T-cell areas. Then within the lymphoid tissue, several members of the tumor necrosis factor (TNF) and TNF-receptor families, such as TRANCE and CD40 ligand, trigger DC production of cytokines like interleukin-12. The TNF family also maintains DC viability. Otherwise the cells die within a day or two. Each of these components of DC function provides targets for manipulating immunity.

Priming of T-cell immunity via dendritic cells

Animal studies

During the early research on DCs, several labs administered antigens to experimental animals and then tried to identify the cells that had captured the antigens in a form that was immunogenic. Regardless of the route of antigen administration (blood, muscle, skin, intestine and airway), DCs were the major reservoir of immunogen.

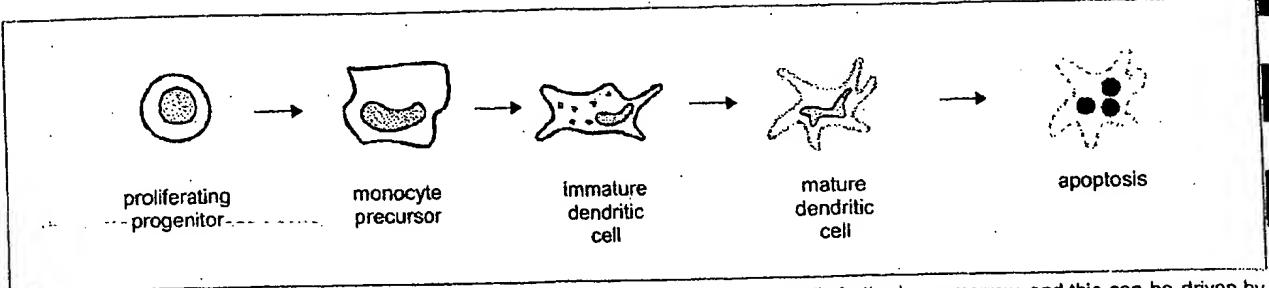


Fig. 3. The life history of dendritic cells. DCs arise from proliferating progenitors, primarily in the bone marrow, and this can be driven by cytokines like Flt-3 ligand and G-CSF. Precursors are formed, such as the monocytes in blood, which then give rise to immature DCs. The immature DCs are capable of producing large amounts of antigen-presenting MHC products and capturing antigens. Multidrug resistance receptors are newly recognized players in the mobilization of immature DCs. DCs mature in response to various stimuli such as infection and inflammation, and migrate under the influence of chemokines to the lymphoid tissues. There the DCs die within a day unless their lifespan is prolonged by TNF-family molecules expressed by the activated T cells.

Next, DCs were used as nature's adjuvant to immunize animals. The DCs were taken from mice or rats, exposed to antigens *ex vivo* and injected back into immunologically naive recipients. The animals became immunized to the antigens that had been captured by the DCs, and the immunization took place in the lymph nodes draining the site of DC injection. Genetic proof was provided that the DCs were priming the animal directly and not simply handing off their antigen to other cells.^{7,8}

DC-based immunization is really very different from all prior attempts at cell therapy. Immunology has had extensive experience with "passive immunization," whereby a recipient is given large numbers of cells that are activated prior to injection. It is hard to produce such large numbers of cells, and their lifespan, diversity and efficacy are all finite. In contrast, when relatively small numbers of antigen-charged DCs are used to induce immunity, this produces "active immunization." Now the animals (and patients, see below) can make their own diverse and longer-lasting immune response to the antigen-bearing DCs.

Human studies

The above experiments made it clear that DCs, pulsed *ex vivo* with antigens, actively immunized animals and raised the exciting possibility that scientists would be able to induce resis-

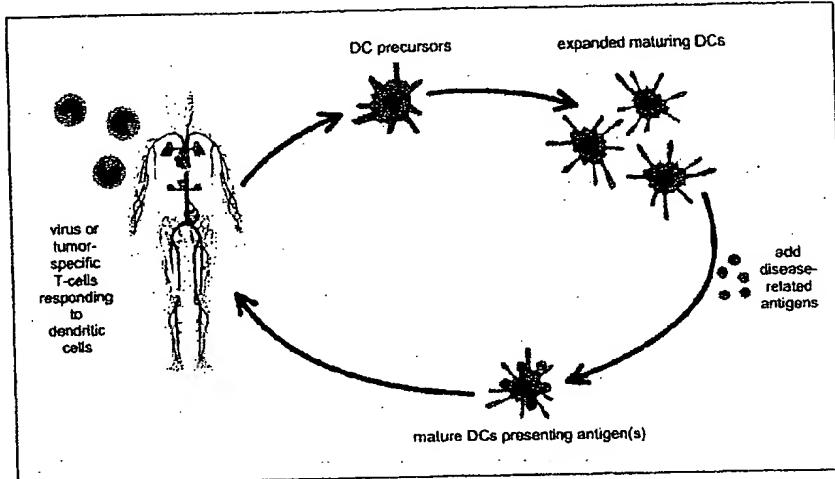


Fig. 4. The use of dendritic cells as adjuvants for enhancing immunity to tumors and infectious agents in humans. This new form of immune therapy begins with the isolation of DC precursors from the patient, usually from blood. The precursors develop *ex vivo* (in relatively simple tissue culture systems) into large numbers of more mature DCs. During this time, the DCs are charged with antigens from the tumor or infection. Then the DCs are reinfused to elicit immunity and thereby resistance to the disease.

tance to tumors, infections and transplants in patients. For example, could one expose patients' DCs *ex vivo* to antigens in their tumors and then reinject the antigen-bearing DCs to elicit tumor-specific immunity (Fig. 4)? This approach is actually not terribly complicated, but one first had to overcome a major obstacle and learn to generate large numbers of DCs. These techniques became available in the 1990s. They have energized the field and, accordingly, clinical trials for the immunization of humans against cancer have begun on most continents.

It is evident that DCs can serve as adjuvants for humans, converting antigens into immunogens.^{9,10} Even in advanced cancer, immune responses already have been observed that are similar to or better than immune responses obtained with other approaches. However, this approach is still in its preliminary stages, since a good deal of science remains to be developed. On the one hand, there are critical unknowns in terms of overall DC biology. Many of the clinical studies to date, for example, have overlooked key features that could improve DC function, such as the need for DCs

TABLE II: DENDRITIC CELL SPECIALIZATION TO INCREASE MHC-PEPTIDE COMPLEX FORMATION

- Receptors for antigen uptake, e.g., DEC-205
- Processing of dying cells, nonreplicating microbes and immune complexes onto MHC class I ("cross-presentation")
- Regulation of antigen processing by maturation stimuli
- Clustering of T-cell receptor ligands with co-stimulators like CD86

to be sufficiently mature (see below) to be effective *in vivo*. Also, DC biology has to be placed in the context of specific tumors and pathogens and patients for DC-based therapies to be optimized.

To summarize and further illustrate the role of DCs in the context of human disease (Table I), consider the need to harness T cells to resist tumors and chronic infections. Protein antigens often are known for a tumor-like melanoma, or for a virus like HIV-1 whose genetic sequence has been available for more than 15 years. However, this knowledge about antigens from melanoma and HIV-1 antigens remains to be converted into methods that provide better immunogens either for immune therapy of melanoma or for the design of HIV-1 vaccines. This is because some important facts of immunological life are being overlooked. When antigens are injected, they also need to gain access to the right DCs to become immunogens (Fig. 1).

Delivering antigens to dendritic cells

Broadly speaking, a central goal is to learn how to deliver or "target" antigens to DCs and simultaneously to differentiate or "mature" the cells to their most potent state. These two challenges, antigen targeting and DC maturation, prove to be intertwined.

Targeting means that the antigen should be in a form that the DCs can recognize. Without such recognition, the uptake and subsequent processing of antigen to form MHC-peptide complexes is suboptimal. DCs have a number of special mechanisms for capturing antigens and converting these into MHC-peptide complexes (Table II). For example, DCs have a receptor called DEC-205 whose binding partners or ligands are still unknown. Nonetheless, it is clear that DEC-205 greatly increases the capacity of DCs to form MHC-peptide complexes.¹¹ DCs also carry out a fascinating process called "cross-presentation." DCs can take up dying cells and effi-

ciently extract peptides from them, so antigens "cross" from the dying cell to the DC. The discoverers of this phenomenon called it "resurrecting the dead."¹² Cross-presentation allows DCs to efficiently form MHC-peptide complexes from dead cells in tumors, transplants and tissues under autoimmune attack.

Special uptake and processing mechanisms allow DCs to tailor a protein antigen, as well as the proteins in a complex microbe or tumor cell, into peptides that bind to an individual's MHC products. The latter are exceptionally polymorphic, differing genetically from one individual to another. As a result, the relevant immunizing peptides differ from one individual to another. One reason why peptides are not ideal immunogens is that they must be individualized. DCs, in contrast, can capture antigens with high efficiency and likewise extract peptides that are relevant for any individual.

A second DC advantage is that these cells have the many required accessory or co-stimulatory properties for converting the selected peptides ("antigens") into effective immunogens. A third DC advantage is that these cells position themselves in a way that leads to the identification of rare antigen-reactive T lymphocytes *in vivo* (Fig. 2). DCs thus overcome many of the difficult obstacles in initiating immunity.

In order for an antigen to be a strong immunogen, one needs to provide a stimulus for the final differentiation or maturation of the DCs (Fig. 3). Most DCs in the body are in an immature state and lack many features that lead to a strong T-cell response.

Immature DCs, for example, lack the CD86 and CD40 molecules that greatly boost the DC-T cell interaction. Immature DCs also lack a chemokine receptor called CCR7 that seems very important for proper migration and homing to lymph nodes to start immunity. For cancer immunology, it is unlikely that tumors provide maturation stimuli. Tumors may even block DC maturation induced by other stimuli. Therefore it is important to learn how to deliver tumor cells to DCs and bypass the normal obstacles to effective antitumor immunity.

Surprising recent evidence actually links DC maturation to the efficient formation of MHC-peptide complexes or TCR ligands (Table II). Immature DCs take up antigens, but they do not make abundant MHC-peptide complexes until they receive a maturation stimulus.^{13,14} Maturation also up-regulates CD86 co-stimulators, but the CD86 actually travels together with the TCR ligands to the surface of the DCs. At the DC surface, the MHC molecules and CD86 remain clustered with each other, keeping the machinery for T-cell activation juxtaposed. This phenomenon will help explain the potency of DCs, because TCR ligands and co-stimulators are displayed together on the cell surface and in high levels.

Control points beyond antigen targeting and maturation of DCs

Research on DCs is moving more vigorously, because the cells are more readily available and because their role in the immune system is considered essential. Nonetheless, researchers in this field are just beginning to find ways to manipulate DCs *in situ*. Putting together an antigen that targets

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to DCs with a stimulus for DC maturation will be a major step in improving the conversion of antigens into immunogens, as in immune-based therapies against tumors and infectious agents.

Additional challenges and questions are evident:

- How can DC numbers be increased *in situ* and how can active DCs be mobilized to a cancer or site of chronic infection?
- Can DCs induce strong immune memory to make vaccination long lasting and effective (we have only been reviewing the role of DCs in the initiation of immunity)?
- Can DCs change the quality of the immune response? "Quality" refers to recent evidence for different types of DCs, especially a subset that induces Th1-type T cells for resistance to infectious agents and strong memory.
- Is it possible to move beyond DC-based immunization experiments and use DCs to either regulate or tolerize the immune system, as frequently required in transplantation and autoimmune diseases?
- Can DCs influence elements of the immune system other than T cells; for example, B cells and the innate defenses provided by natural killer (NK) and NK-T cells?

The answer to all these questions is a preliminary "yes." As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent.

Dendritic cells and better control of disease

DCs provide important avenues for the investigation of human disease. Many labs are exploiting DCs to identify antigens relevant for immunity against human pathogens. In these experiments, one introduces complex but clinically important antigens to DCs and then identifies which components are best presented to the immune system. We have recently used this approach to identify previously un-

known immune responses to the Epstein-Barr virus,¹⁵ a virus we all carry that has the potential to cause cancer like Hodgkin's lymphoma. Other laboratories have been using DCs to identify new antigens in other infectious agents, in transplants and in cancers like melanoma.

Investigators are also manipulating DCs *ex vivo* and then reinfusing the cells to identify conditions leading to strong immunity in patients (Fig. 4). In particular, DC-mediated active immunization against cancer is being vigorously pursued, as mentioned above. Instead of manipulating DCs *ex vivo*, a more desirable goal would be able to alter DCs directly *in situ*. Some approaches are under way. An example is the injection of cytokines like flt3 ligand and G-CSF to mobilize various precursor populations of DCs. One should also develop methods to control DC mobilization, migration and maturation. In sum, DCs are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

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Ralph M. Steinman, M.D., is Henry G. Kunkel Professor and Senior Physician, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, 1230 York Ave., New York, New York 10021-6399, U.S.A.

Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor

(heterodimeric lymphokine/T-cell growth factor/lymphokine-activated killer cells/coordinate gene regulation/interleukin-12)

UELI GUBLER*†, ANNE O. CHUA*, DAVID S. SCHOENHAUT*, CYNTHIA M. DWYER‡, WARREN MCCOMAS*, RICHARD MOTYKA*, NASRIN NABAVI§, AIMEE G. WOLITZKY§, PHYLLIS M. QUINN§, PHILIP C. FAMILLETTI‡, AND MAURICE K. GATELY§

Departments of *Molecular Genetics, †Bioprocess Development, and §Immunopharmacology, Roche Research Center, Hoffmann-La Roche, Inc., Nutley, NJ 07110

Communicated by John J. Burns, February 4, 1991

ABSTRACT Cytotoxic lymphocyte maturation factor (CLMF) is a disulfide-bonded heterodimeric lymphokine that (i) acts as a growth factor for activated T cells independent of interleukin 2 and (ii) synergizes with suboptimal concentrations of interleukin 2 to induce lymphokine-activated killer cells. We now report the cloning and expression of both human CLMF subunit cDNAs from a lymphoblastoid B-cell line, NC-37. The two subunits represent two distinct and unrelated gene products whose mRNAs are coordinately induced upon activation of NC-37 cells. Coexpression of the two subunit cDNAs in COS cells is necessary for the secretion of biologically active CLMF; COS cells transfected with either subunit cDNA alone do not secrete bioactive CLMF. Recombinant CLMF expressed in mammalian cells displays biologic activities essentially identical to natural CLMF, and its activities can be neutralized by monoclonal antibodies prepared against natural CLMF. Since this heterodimeric protein displays the properties of an interleukin, we propose that CLMF be given the designation interleukin 12.

The molecular cloning and expression of recombinant cytokines has made possible both significant advances in our understanding of the molecular basis of immune responses and the development of new approaches to the treatment of disease states. As an example, recombinant interleukin 2 (recombinant IL-2) has been shown to be capable of causing regression of established tumors in both experimental animals (1) and in man (2); however, its clinical use has been associated with significant toxicity (2). One potential approach to improving the therapeutic utility of recombinant cytokines is to use them in combination (3, 4). With this concept in mind, we initiated a search for novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes *in vitro* and thus might have synergistic immunoenhancing effects when administered together with recombinant IL-2 *in vivo*. This led to the identification of a factor, designated cytotoxic lymphocyte maturation factor (CLMF), that synergized with recombinant IL-2 to facilitate the generation of both cytolytic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells *in vitro* (5, 6). CLMF was subsequently purified to homogeneity from a human lymphoblastoid B-cell line (NC-37) and was shown to be a 75-kDa disulfide-bonded heterodimer composed of two subunits with molecular masses of 40 kDa and 35 kDa (7).[¶] We now report the molecular cloning and expression of CLMF.

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MATERIALS AND METHODS

cDNA Cloning. A subline of NC-37 cells selected for its ability to produce high levels of CLMF (7), NC-37.98, was induced with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 for 16 hr. Poly(A)⁺ RNA was isolated, and random hexamer-primed cDNA libraries were established in phage λgt10 by standard procedures. Mixed-primer polymerase chain reaction (PCR) using controlled ramp times (8) was performed as follows. PCR primers contained all possible codons and were 14 or 15 nucleotides long (Fig. 1) with a 5' extension of 9 nucleotides containing an EcoRI site for subcloning. Degeneracies varied from 1 in 32 to 1 in 4096; 0.5–4 pmol per permutation of forward and reverse primer was used in a 50- to 100-μl PCR mixture with 40 ng of cDNA made from NC-37.98 cells that had been activated by culture with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml for 16 hr (40-kDa subunit) or with 3 μg of human genomic DNA (35-kDa subunit). PCR cycling parameters were as follows. Initial denaturation was at 95°C for 7 min. Low-stringency annealing was performed by cooling to 37°C over 2 min, incubating 2 min at 37°C, heating to 72°C over 2.5 min, extending at 72°C for 1.5 min, heating to 95°C over 1 min, and denaturing at 95°C for 1 min. This cycle was repeated once. Thirty standard cycles (40-kDa subunit) or 40 standard cycles (35-kDa subunit) were performed as follows: 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min. Final extension was at 72°C for 10 min. "Amplicons" of the expected size were gel-purified, subcloned, and sequenced. The 40-kDa subunit cDNAs were isolated by hybridizing the 54-mer amplicon in 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 20% formamide at 37°C overnight. Filters were washed in 2× SSC at 42°C for 30 min and exposed to x-ray film. The 35-kDa subunit cDNAs were isolated by hybridizing the 51-mer amplicon in 5× SSC/20% formamide at 37°C overnight. The filters were washed in 2× SSC at 40°C for 30 min and exposed to x-ray film. Positive clones were plaque-purified, their inserts were subcloned into the pBluescript plasmid, and their sequences were determined by using Sequenase.

Expression. cDNAs were separately engineered for expression in vectors containing the simian virus 40 early promoter essentially as described (9). COS cells were transfected with both CLMF subunit expression plasmids mixed together or

Abbreviations: CLMF, cytotoxic lymphocyte maturation factor; rCLMF and nCLMF, recombinant and natural CLMFs; CTL, cytolytic T lymphocyte; IL, interleukin; LAK, lymphokine-activated killer; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; n, natural; PCR, polymerase chain reaction.

[†]To whom reprint requests should be addressed.

[¶]The cDNA sequences reported in this paper have been deposited in the Genbank data base [accession nos. M38443 (35-kDa CLMF subunit) and M38444 (40-kDa CLMF subunit)].

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1 MCPARSLLL V ATLVLLDHLS LARNLPVATP DPGMFPCCLHH SQNLLRAVSN
51 MLQKARQTLE FYPCTSEEID HEDITDKTS TVEACLPLEL TKNESCLNSR
101 ETSFITNGSC LASRKTSFMM ALCLSIIYED LKMYQVEFKT MNAKLLMDPK
151 RQIFLDQNML AVIDELMQAL NFNSETVPQK SSLEEPDFYK TKIKLCILLH
201 AFRIRAVTID RVTSYLNAS

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1 MCHQQQLVISW FSLVFLASPL VAIWELKKDV YVVELDWYPD APGEMVVLTC
51 DTPEEDGITW TLDQSSEVLG SGKTLTIQVK EFGDAGQYTC HKGGEVLSHS
101 LLLLHKKEDG IWSTDILKDQ KEPKNKTFLR CEANKNSGRF TCWWLTTIST
151 DLTFSVKSSR GSSDPQGVTC GAATLSAERV RGDNKEYEYS VECQEDSACP
201 AAEESLPIEV MVDAVHKLKY ENYTSSFFIR DIIKPDPPKN LQLKPLKNSR
251 QVEVSWEYPD TWSTPHSYFS LTFCVQVQGK SKREKKDRVF TDKTSATVIC
301 RKNASISVRA QDRYYSSWS EWASVPCS

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FIG. 1. Amino acid sequences of the 35-kDa (*Upper*) and 40-kDa (*Lower*) CLMF subunits as deduced from the respective cDNAs and shown in single-letter code. Signal peptides are overlined, cysteine residues are marked by a caret, and N-linked glycosylation sites (NXS, NXT, where X is another amino acid) are underlined. The peptide sequences used to generate PCR probes are overlined with arrows indicating the direction of amplification.

with each one separately by the DEAE-dextran method. Twenty-four hours after transfection, the serum-containing medium was replaced with medium containing 1% Nutridoma-SP (Boehringer Mannheim), and supernatant fluids were collected from the cultures after 40 hr. These fluids were stored at 4°C until tested in the bioassays.

General Methods. Standard molecular biological procedures were used as described (10). CLMF bioassays were performed as detailed (7).

Computer Searches. The National Biomedical Research Foundation protein data base (Release 26.0) as well as the Genbank and European Molecular Biology Laboratory databases (Releases 65.0 and 24.0, respectively) were searched for sequences homologous to CLMF cDNAs. The two subunit sequences were compared to each other using the ALIGN program (mutation data matrix, break penalty of 6; see ref. 11).

RESULTS

Partial N-terminal amino acid sequences of the two CLMF subunits (7) were used to generate completely defined 51- to 54-base-pair (bp)-long oligonucleotide probes by means of mixed primer PCR. These probes were used to screen cDNA libraries made from RNA from activated NC-37.98 cells, and cDNAs encoding the two subunits were isolated and characterized. Both cDNAs encode secreted proteins with a classical hydrophobic N-terminal signal peptide immediately followed by the N terminus of the mature protein as determined by protein sequencing (7). Two independent cDNA clones for the 40-kDa subunit were shown to be identical. Both encode the mature 40-kDa subunit that is composed of 306 amino acids (calculated $M_r = 34,699$) and contains 10 cysteine residues and four potential N-linked glycosylation sites (Fig. 1). Two of these sites are within isolated tryptic peptides derived from the purified 40-kDa CLMF subunit protein. Amino acid sequence analysis has shown that Asn-

222 is glycosylated, whereas Asn-125 is not (Fig. 1; F. Podlaski, personal communication). The mature 35-kDa subunit is composed of 197 amino acids (calculated $M_r = 22,513$), with 7 cysteine residues and three potential N-linked glycosylation sites (Fig. 1). When purified CLMF is reduced with 2-mercaptoethanol and analyzed by SDS/PAGE, the 35-kDa subunit appears to be heterogeneous, suggesting that it may be heavily glycosylated (7). Two variants of 35-kDa subunit-encoding cDNAs were isolated. The first type had the sequence shown in Fig. 1. Additional isolates contained what is probably an allelic variation, replacing Thr-213 with a methionine residue.

Computer searches of sequence databases showed that the amino acid sequences of the two subunits are not related to any known protein. The subunit sequences are also not related to each other, since a comparison using the ALIGN program (11) gave a score of 1.27; only scores >3 are considered to indicate significant evolutionary relationship (12). The genes encoding the subunits appear to be unique, since low- and high-stringency hybridizations of genomic blots revealed identical banding patterns (data not shown). RNA blots showed the size of the 40-kDa subunit mRNA to be 2.4 kb, whereas the 35-kDa subunit was encoded by a 1.4-kb transcript (Fig. 2). Expression of the two mRNAs encoding the subunits was coordinately regulated upon induction (Fig. 2). When NC-37.98 cells were activated with PMA and calcium ionophore for 72 hr, mRNA encoding each of the CLMF subunits was minimally detectable at 6 hr after the beginning of induction but was readily detected at 24 hr and continued to accumulate until maximal levels were reached at 72 hr (normalized to GAPDH mRNA levels; see the legend to Fig. 2). In contrast, the mRNA for IL-2 in activated NC-37.98 cells was already at high levels at 6 hr and subsequently decreased, whereas the mRNAs for the low-affinity IL-2 receptor (p55) followed the induction pattern seen for the CLMF subunits. Scanning of RNA blots also revealed that steady-state mRNA levels for the 40-kDa

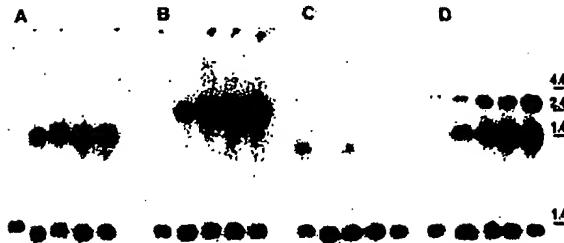


FIG. 2. RNA blots showing the coordinate induction of the 35-kDa (**A**) and 40-kDa (**B**) CLMF subunit mRNAs and IL-2 mRNA (**C**) and its p55 receptor mRNA (**D**). Poly(A)⁺ RNA (5 µg) from NC-37/98 cells activated with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml were loaded in each lane. Lanes from left to right in each panel show RNAs isolated 6, 24, 30, 48, and 72 hr after induction, respectively. (**Upper**) Four-day exposures. (**Lower**) Two-hour exposure of the same blots after stripping and rehybridization with a GAPDH probe. Marker sizes are in kb (BRL RNA ladder).

CLMF subunit were severalfold higher than those for the 35-kDa subunit expressed by the same cells. This finding parallels the observation that activated NC-37 cells secrete excess free 40-kDa subunit protein (7). The 3' untranslated sequences of both CLMF subunit mRNAs contain several copies of the octamer motif TTATTAT (data not shown). This sequence is present in other transiently expressed mRNAs and is involved in regulating mRNA stability (13).

Coexpression of the 40-kDa and 35-kDa CLMF subunit cDNAs in COS cells was required to generate secreted biologically active CLMF (Table 1 and Fig. 3). COS cells transfected with cDNA encoding either the 40-kDa subunit alone or the 35-kDa subunit alone did not secrete biologically active CLMF (Table 1). Mixing media conditioned by COS

cells that had been separately transfected with one or the other of the two CLMF subunit cDNAs also did not give rise to bioactive CLMF (Table 1).

Two types of assays were used to compare rCLMF and nCLMF. The first assay measures the proliferation of phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes, whereas the second assay evaluates the synergy between CLMF and suboptimal concentrations of IL-2 in the generation of LAK cells in hydrocortisone-containing cultures (7). The data in Fig. 3 show that rCLMF as expressed in COS cells and nCLMF as purified from NC-37 cells are essentially identical. Dose-response curves for rCLMF and nCLMF were superimposable in each of the two assays, and rCLMF was neutralized by a monoclonal antibody raised against nCLMF. Conditioned media from cultures of mock-transfected COS cells displayed no activity in these assays (Table 1 and data not shown).

DISCUSSION

In a previous report (7), we described the purification of a heterodimeric cytokine, CLMF, that synergized with low amounts of IL-2 to cause the generation of LAK cells in the presence of hydrocortisone and stimulated the proliferation of activated T cells independent of IL-2. In the present report, we have used the N-terminal amino acid sequence information previously obtained to clone the two subunit cDNAs of CLMF. Protein purification of NC-37 cell line-derived CLMF had shown that the protein was composed of two disulfide-bonded subunits with different N-terminal amino acid sequences (7). However, it was not clear from our previous results whether the two subunits were processed from one common gene product and whether proteolytic posttranslational processing other than signal peptide cleavage was occurring. The molecular cloning and sequencing of

Table 1. Coexpression of both CLMF subunit cDNAs is required for secretion of biologically active CLMF by COS cells

Addition	Conc., units/ml	Dilution	[³ H]Thymidine incorporated by PHA-activated lymphoblasts, mean cpm ± 1 SEM
Cytokine*			
None	—		11,744 ± 514
nCLMF	200		68,848 ± 878
nCLMF	40		48,827 ± 605
nCLMF	8		26,828 ± 594
nCLMF	1.6		17,941 ± 196
Culture fluid from COS cells transfected with			
A. 35-kDa CLMF subunit cDNA		1:20	11,912 ± 660
		1:100	10,876 ± 232
B. 40-kDa CLMF subunit cDNA		1:20	11,699 ± 931
		1:100	11,666 ± 469
C. 35-kDa + 40-kDa CLMF subunit cDNAs		1:20	58,615 ± 587
		1:100	38,361 ± 828
1:1 mix of culture fluids A and B		1:10†	11,544 ± 483
		1:50	10,503 ± 259
CM from mock-transfected control‡		1:20	11,503 ± 286
		1:100	10,751 ± 303

PHA-activated lymphoblasts were prepared from human peripheral blood mononuclear cells as described (7). Lymphoblast proliferation was measured in a 48-hr assay (7) in which 2×10^4 lymphoblasts were incubated in 100-µl cultures containing the indicated amounts of natural CLMF (nCLMF) or COS cell culture fluids. [³H]Thymidine was added to each culture 18 hr prior to harvest. Conc., concentration.

*nCLMF is purified NC-37-derived CLMF.

†1:10 dilution of the 1:1 mixture of culture fluids A and B was equivalent to a 1:20 final dilution of each of the individual culture fluids.

‡Conditioned medium (CM) from cultures of mock transfected COS cells.

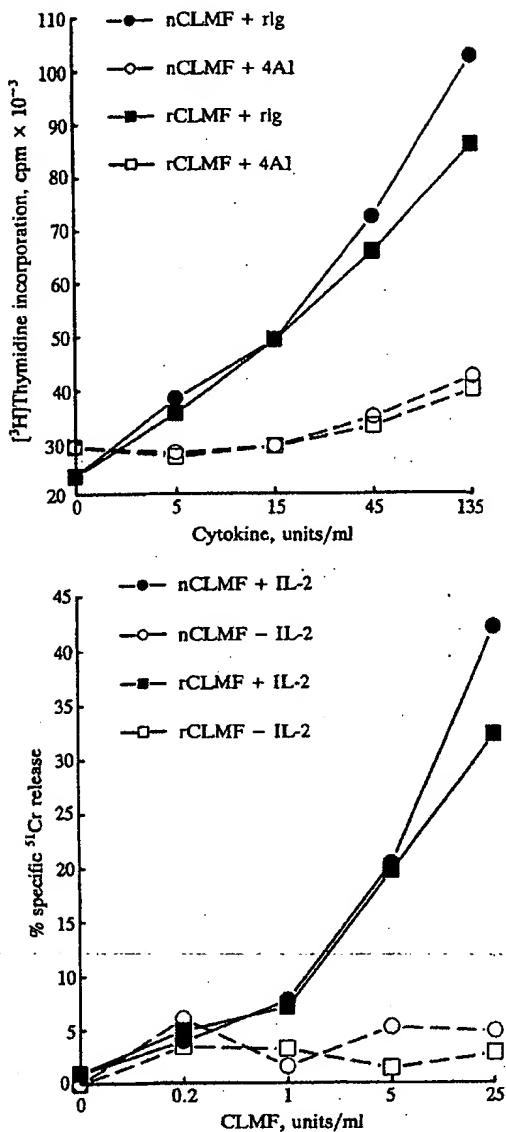


Fig. 3. Comparison of biologic activities of nCLMF (circles) and recombinant CLMF (squares). nCLMF was purified from NC-37 cell-conditioned media; rCLMF was purified from conditioned media from cultures of COS cells transfected with cDNAs encoding the 40-kDa and 35-kDa human CLMF subunits. (*Upper*) T-cell growth factor assay. The ability of CLMF to stimulate the proliferation of human PHA-activated lymphoblasts in 48-hr cultures was assayed as described (7). CLMF preparations were mixed with neutralizing rat monoclonal anti-human CLMF antibody 4A1 (ref. 7; open symbols) or with normal rat IgG (Sigma; IgG, closed symbols) at a final concentration of 20 μ g of IgG/ml and were incubated for 30 min at 37°C prior to addition of PHA blasts. All values are means of triplicate determinations. (*Lower*) LAK cell induction assay. The ability of CLMF, alone or in combination with recombinant IL-2, to induce the generation of LAK cells in 4-day cultures was assessed as described (7). Low-density peripheral blood lymphocytes were incubated in the presence of various amounts of nCLMF or rCLMF with (closed symbols) or without (open symbols) recombinant IL-2 at 7.5 units/ml. Units of CLMF activity were based on previous titrations in the T-cell growth factor assay. Hydrocortisone sodium succinate (Sigma) was included at a concentration of 0.1 mM to minimize triggering of endogenous cytokine cascades. Lysis of ^{31}Cr -labeled Daudi cells was assessed at an effector/target ratio of 5:1. The data shown represent the means of quadruplicate determinations. The spontaneous ^{31}Cr release was 20%.

the corresponding cDNAs now has demonstrated that there is no common precursor for the two CLMF subunits; rather, they are encoded by completely different genes. The predicted and actual amino acid composition for the two subunits are strikingly similar; differences in predicted versus actual molecular weights are accounted for by glycosylation (F. Podlaski, personal communication). Thus, the only major posttranslational proteolytic event that appears to take place in the maturation of the CLMF subunits is signal peptide cleavage.

The kinetics of expression of the individual CLMF subunit mRNAs were examined and compared to the expression of mRNAs for IL-2 and the IL-2 receptor p55. Previously it had been observed that NC-37 cells, like certain murine (14) and marmoset (15) B-cell lines, secreted IL-2 when activated (M.K.G., unpublished results). RNA blots demonstrated that upon activation of NC-37 cells, both CLMF subunit mRNAs were coordinately induced with kinetics similar to the IL-2 receptor (p55) mRNAs. On the other hand, IL-2 mRNA levels peaked much earlier. Similar differences in induction kinetics were also seen at the level of IL-2 and CLMF bioactivity secreted from NC-37 cells (M.K.G., unpublished data). These kinetic differences are consistent with our previous observation that in a cytolytic lymphocyte response, IL-2 appears to act earlier than CLMF (5).

Transfection studies with COS cells established that only coexpression of both subunit cDNAs gives rise to secreted bioactive CLMF. Thus, it appears that the two proteins have to interact within the endoplasmic reticulum to assemble properly into bioactive secreted CLMF. By comparing the activity of rCLMF to that of nCLMF in the T-cell growth factor and LAK cell induction assays (Fig. 3) and assuming that the specific activity of rCLMF is similar to that of nCLMF [8×10^7 units/mg (7)], we estimate that the amount of rCLMF heterodimer produced in these experiments was 5–50 ng/ml. The finding that COS cells, which are fibroblast-like cells, are able to assemble correctly the two CLMF subunits to form bioactive CLMF indicates that this secretion and processing pattern is not limited to cells of the lymphoid lineage.

Western blot analysis using an anti-CLMF antibody specific for the 40-kDa subunit has allowed confirmation that (*i*) COS cells transfected with both CLMF subunit cDNAs secrete CLMF with the expected heterodimeric structure and (*ii*) COS cells transfected with the 40-kDa subunit cDNA alone secrete that subunit (F. Podlaski, personal communication). Since no bioactivity was detected in media conditioned by COS cells transfected with only the 40-kDa subunit, that subunit by itself appears either to have a much reduced specific activity compared with heterodimeric CLMF or to be completely inactive.

Because of the lack of a high-affinity antibody specific for the 35-kDa subunit, we have not yet been able to determine definitively whether COS cells transfected with only the 35-kDa subunit cDNA secrete that subunit. Since no bioactivity was detected in the media, secretion of a bioactive 35-kDa subunit by itself could be very inefficient; alternatively, similar to the 40-kDa subunit, the protein could be much less active or inactive altogether. Intracellular 35-kDa protein in the absence of the other subunit could be inherently unstable; there is precedence for this phenomenon, since it has been reported that 90% of the β chains of lutropin (LH), when expressed in the absence of α chains, are retained in the endoplasmic reticulum and are slowly degraded (16). Simple mixing of media conditioned by COS cells transfected separately with either one of the two CLMF subunit cDNAs did not yield bioactive CLMF. One possible explanation would be that the cells do not secrete the 35-kDa CLMF subunit by itself. More likely, our experimental conditions did not allow proper heterodimer formation. One would expect that only

carefully controlled renaturation and oxidation conditions would allow the disulfide bond formation required for generation of bioactive CLMF.

Normal human peripheral blood lymphocytes under the appropriate induction conditions produce both CLMF subunit mRNAs and secrete the active protein (N.N. and M.K.G., unpublished data). There is some evidence suggesting that CLMF is produced predominantly by B cells. In preliminary experiments, B-cell mitogens have appeared to be more effective than T-cell mitogens in eliciting CLMF production from peripheral blood lymphocytes (M.K.G., unpublished results). When screening human cell lines for their ability to produce CLMF activity (7), we observed that four of eight B-cell lines tested produced CLMF after activation with PMA and calcium ionophore, whereas none of five T-cell lines produced CLMF. Nevertheless, three of these T-cell lines secreted large amounts of IL-2 and tumor necrosis factor activity after activation (M.K.G., unpublished results). Likewise, natural killer cell stimulatory factor (NKSF), a heterodimeric cytokine similar or identical to CLMF, was isolated from RPMI 8866 lymphoblastoid B cells (17). A recent report (18) has indicated that B lymphocytes can secrete a cytokine(s) distinct from IL-2 that facilitates virus-specific cytolytic T-lymphocyte responses. It is possible that CLMF may have been the cytokine active in those studies. Thus, although B lymphocytes have not traditionally been viewed as cytokine-producing helper cells, it is conceivable that CLMF production constitutes a novel mechanism whereby B lymphocytes contribute to the amplification of T-lymphocyte responses. In addition to the biologic activities described in this report, CLMF by itself has been shown (i) to activate NK cells in an 18–22 hr assay, (ii) to facilitate the generation of specific allogeneic CTL responses, and (iii) to stimulate the secretion of γ interferon by resting peripheral blood lymphocytes (M.K.G., unpublished results). It can also synergize with low concentrations of recombinant IL-2 in the latter two assays and in causing the proliferation of resting peripheral blood lymphocytes. In view of its production by peripheral blood lymphocytes and its diverse actions on lymphoid cells, it appears that CLMF constitutes a new interleukin. We propose that CLMF be

given the provisional designation IL-12. The availability of recombinant CLMF will now make possible a broader and more detailed characterization of its biology.

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Immunization With Melan-A Peptide-Pulsed Peripheral Blood Mononuclear Cells Plus Recombinant Human Interleukin-12 Induces Clinical Activity and T-Cell Responses in Advanced Melanoma

By Amy C. Peterson, Helena Harlin, and Thomas F. Gajewski

Purpose: Preclinical studies showed that immunization with peripheral blood mononuclear cells (PBMC) loaded with tumor antigen peptides plus interleukin-12 (IL-12) induced CD8⁺ T-cell responses and tumor rejection. We recently determined that recombinant human (rh) IL-12 at 30 to 100 ng/kg is effective as a vaccine adjuvant in patients. A phase II study of immunization with Melan-A peptide-pulsed PBMC + rhIL-12 was conducted in 20 patients with advanced melanoma.

Patients and Methods: Patients were HLA-A2-positive and had documented Melan-A expression. Immunization was performed every 3 weeks with clinical re-evaluation every three cycles. Immune responses were measured by ELISpot assay before and after treatment and through the first three cycles, and were correlated with clinical outcome.

Results: Most patients had received prior therapy and had visceral metastases. Nonetheless, two patients achieved a

complete response, five patients achieved a minor or mixed response, and four patients had stable disease. The median survival was 12.25 months for all patients and was not yet reached for those with a normal lactate dehydrogenase. There were no grade 3 or 4 toxicities. Measurement of specific CD8⁺ T-cell responses by direct ex vivo ELISpot revealed a significant increase in interferon gamma-producing T cells against Melan-A ($P = .015$) after vaccination, but not against an Epstein-Barr virus control peptide ($P = .86$). There was a correlation between the magnitude of the increase in Melan-A-specific cells and clinical response ($P = .046$).

Conclusion: This immunization approach may be more straightforward than dendritic cell strategies and seems to have clinical activity that can be correlated to a biologic end point.

J Clin Oncol 21:2342-2348. © 2003 by American Society of Clinical Oncology.

MOST MELANOMA tumors express antigens that can be recognized by CD8⁺ T cells.^{1,2} Nonetheless, tumors frequently escape immune destruction, either from a failure to generate an optimal tumor antigen-specific T-cell response or from development of resistance to the T-cell response induced. One strategy to overcome the former hurdle is through active immunization, the opportunity for which has been facilitated by the molecular definition of melanoma antigens.³ Specific CD8⁺ T cells that are properly activated can home to tumor sites and kill tumor cells, to the extent to which they can overcome negative immunoregulatory pathways and tumor resistance.⁴

The optimal immunization strategy for inducing tumor antigen-specific CD8⁺ effector T cells in humans remains undefined. However, antigen-presenting cell-based strategies have shown promise. Both monocyte-derived^{5,6} and bone marrow-derived⁷ dendritic cells (DCs) have been loaded with

melanoma tumor antigens and administered in the advanced-disease setting, with evidence for immunization and tumor regression in subsets of patients. However, DCs are cumbersome to generate and alternative approaches that are more straightforward yet equally as effective would be useful. One cofactor produced by DCs that contributes to their efficacy is interleukin-12 (IL-12), which facilitates the induction of interferon gamma (IFN- γ)-producing cytolytic effector cells.⁸⁻¹⁰ Endogenous IL-12 seems necessary for optimal rejection of immunogenic murine tumors^{11,12} and provision of exogenous IL-12, either alone¹³ or combined with tumor antigen-based vaccines,¹⁴⁻¹⁹ can induce rejection of pre-established tumors in murine models. We previously have shown that coadministration of IL-12 with peripheral blood mononuclear cells (PBMCs) loaded with tumor antigen peptides induced specific cytolytic T-lymphocyte responses and tumor protection in mice, circumventing the need to generate dendritic cells.²⁰ The ease by which PBMC can be isolated from patients has made this an attractive approach for clinical translation. We recently conducted a phase I clinical study to determine the dose of recombinant human (rh) IL-12 necessary to induce T-cell responses in combination with antigen-loaded PBMCs, and found that doses from 30 to 100 ng/kg administered subcutaneously (sc) at the vaccine site were optimal and well tolerated.²¹ The effective range of doses indicated that a straight dose of 4 μ g might be used.

In this article, we describe results of a phase II clinical study of immunization with Melan-A/MART-1³ peptide-pulsed autologous PBMCs + rhIL-12 in HLA-A2-positive patients with

From the University of Chicago, Departments of Pathology and Medicine, Section of Hematology/Oncology, and the Ben May Institute for Cancer Research, Chicago, IL.

Submitted December 26, 2002; accepted March 26, 2003.

Supported by the Burroughs Wellcome Fund, Research Triangle Park, NC, and the Cancer Research Institute, New York, NY.

A.P. and H.H. contributed equally to this work.

Address reprint requests to Thomas F. Gajewski, MD, PhD, University of Chicago, 5841 S. Maryland Ave., MC2115, Chicago, IL 60637; email: tgajewsk@medicinebsd.uchicago.edu.

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0732-183X/03/2112-2342/\$20.00

advanced melanoma. Immune responses were analyzed using a direct ex vivo ELISpot assay. We show that this vaccine approach had clinical activity and that the magnitude of increased T-cell response correlated with clinical outcome.

PATIENTS AND METHODS

Patient Enrollment and Eligibility

This was an open-label, nonrandomized, single-institution study of Melan-A peptide-pulsed autologous PBMCs + rhIL-12.⁴ The protocol was approved by the University of Chicago Institutional Review Board and all patients signed written informed consent. Patients who were both HLA-A2-positive and showed Melan-A tumor expression by reverse transcriptase polymerase chain reaction (RT-PCR) were considered for inclusion. Additional inclusion criteria were life expectancy more than 12 weeks, Karnofsky performance status ≥ 70 , and adequate hematopoietic, renal, and hepatic function. Delayed-type hypersensitivity (DTH) skin testing was performed against mumps, *Candida*, and *Trichophyton*, not for eligibility but to correlate subsequently with clinical outcome and immunization potential. Patients were excluded if they had severe cardiovascular disease or arrhythmia, were pregnant or nursing, had biologic therapy received within 4 weeks, tested positive for hepatitis B surface antigen or human immunodeficiency virus (HIV), had clinically significant autoimmune disease or any illness requiring immunosuppressive therapy, had a psychiatric illness that would interfere with patient compliance and informed consent, had active gastrointestinal bleeding or uncontrolled peptic ulcer disease, or had uncontrolled brain metastases. Patients with treated brain metastases who were clinically and radiographically stable and did not require corticosteroids were allowed to enter onto the trial.

Patient Characteristics

Twenty patients with metastatic melanoma were enrolled after giving written informed consent. Patient characteristics are outlined in Table 1. All patients had advanced disease; the majority had at least three sites of metastasis, 60% of which were visceral (ie, noncutaneous and nonpulmonary metastases). Approximately two thirds of the patients had received prior therapy, and 10 patients had an elevated lactate dehydrogenase (LDH) level, which is an important negative prognostic factor.²² Only 45% were positive for at least one recall antigen (mumps, *Candida*, or *Trichophyton*) by DTH skin testing.

RT-PCR Analysis

RNA was isolated from fresh tumor cells using guanidine and cesium chloride. cDNA was synthesized and PCR was performed for Melan-A and beta-actin using the primer pairs and reaction conditions described previously.²¹ Control reactions without reverse transcriptase were performed to rule out a contribution of genomic DNA. PCR products were visualized using a 1.5% ethidium bromide-stained agarose gel. No formal quantitation was performed.

Vaccine Preparation

Therapy consisted initially of three 21-day cycles. Vaccinations were given on the first day of each cycle and rhIL-12 was administered subcutaneously on days 1, 3, and 5. Approximately 100 to 150 mL of peripheral blood from patients was collected on day 1 of each cycle into heparinized 60-mL syringes using sterile technique. PBMCs were isolated over a Lymphoprep gradient (Lymphoprep; Axis-Shield PoC, Oslo, Norway), counted, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) at 40×10^6 cells/mL. At least 10×10^6 cells from each sample were cryopreserved to prepare CD8⁺ and CD8⁻ fractions for subsequent correlative immunologic studies. The Melan-A₂₇₋₃₅ peptide (AAGIGILTV) was produced according to good manufacturing practice standards by Multiple Peptide Systems (San Diego, CA) and provided in lyophilized vials. Aliquots of peptide were prepared at 5 mmol/L in dimethyl sulfoxide and stored at

Table 1. Patient Characteristics

Patient Characteristic	No.	Patients (n = 20)
	No.	%
Age, years		
Median	58	
Range	35-79	
Sex		
Male	9	45
Female	11	55
Karnofsky performance status (ECOG)		
90%-100% (0)	10	50
70%-80% (1)	9	45
60%-70% (2)	1	5
No. of metastatic sites		
1	2	10
2		None
≥3	18	90
Location of metastases		
Visceral	13	65
Brain (treated)	4	20
Prior therapy		
None	6	30
Chemotherapy or immunotherapy	7	35
As only prior therapy	5	25
Chemotherapy	1	5
As only prior therapy	1	5
Immunotherapy	4	20
As only prior therapy	1	5
Other*	2	10
As only prior therapy		None
Adjuvant IFN-α	5	25
As only prior therapy	3	15
Elevated LDH	10	50
DTH recall positive	9	45

Abbreviations: ECOG, Eastern Cooperative Oncology Group; IFN-α, interferon alfa-2b; LDH, lactate dehydrogenase; DTH, delayed-type hypersensitivity.

*Experimental therapy other than a melanoma vaccine, immunomodulatory cytokine, or chemotherapy.

-80°C for up to 3 months. Peptide preparations were quality controlled for HLA-A2 binding, sterility, and identity by high-performance liquid chromatography and mass spectrometry. An aliquot of peptide was diluted to 20 μmol/L in DPBS and mixed with an equal volume of patient PBMCs (final peptide concentration, 10 μmol/L; target number of PBMCs, 10⁸) followed by incubation at 37°C for 1 hour in 10 mL DPBS. The cells were then irradiated (20 Gy), washed in DPBS, and resuspended in 1 mL DPBS. The suspension of peptide-loaded PBMCs was injected sc using a 1-mL syringe and a 21-gauge needle, divided evenly into two sites. Preferred sites were those near draining lymph node basins but not near a tumor mass. The actual number of PBMCs administered per vaccine ranged from 78 to 100 × 10⁶.

rhIL-12 was provided by Genetics Institute (Cambridge, MA) as a lyophilized powder of 10 μg under vacuum. Each vial was intended for single use only and was stored as a powder in our research pharmacy at 2 to 8°C until reconstituted with sterile water for injection. Once reconstituted, rhIL-12 was loaded into 3-mL syringes and used within 4 hours. rhIL-12 (4 μg) was administered sc with a 25-gauge needle just after pulsed PBMC inoculation and immediately adjacent to one of the two immunization sites on days 1, 3, and 5. The same approximate location was used for each injection of peptide-pulsed PBMCs and rhIL-12 for each cycle.

Toxicity Assessment and Criteria for Clinical Response

Toxicities were determined using the National Cancer Institute common toxicity criteria scale version 2.0. A complete response (CR) was assigned if there was disappearance of all lesions without the appearance of any new

lesions; a partial response (PR) was defined as $\geq 50\%$ reduction in total tumor volume; a minor response (MR) was defined as less than 50% reduction in total tumor volume; progressive disease (PD) was assigned if new lesions appeared, any tumor reappeared, or if a 25% increase in tumor area was observed; a mixed response was assigned if at least one tumor decreased in size with other or new tumors growing; stable disease (SD) was anything that did not fit the aforementioned criteria. When possible, cutaneous lesions were photographed.

CD8⁺ T-Cell Preparation

CD8⁺ and CD8⁻ fractions from PBMC were isolated at the time of preparation of each vaccine and cryopreserved until analysis in batch fashion. CD8⁺ T lymphocytes were isolated by positive selection using CD8 microbeads and magnetic columns (MACS system; Miltenyi Biotech, Auburn, CA). The unbound CD8⁻ fraction was cryopreserved for use as antigen-presenting cells for in vitro expansion of specific CD8⁺ T cells. Although the primary ELISpot analysis was performed directly with thawed cells, a secondary assay was carried out after in vitro expansion. For in vitro expansion, CD8⁻ cells were thawed from each time point and pooled, pulsed with 50 $\mu\text{mol/L}$ Melan-A peptide in serum-free Iscove's modified Dulbecco's medium (IMDM) with beta₂-microglobulin, irradiated (3,000 rad), washed, and plated at 2×10^6 cells/well in 24-well plates. CD8⁺ T cells were thawed and cultured with the irradiated CD8⁻ cells at 4×10^5 cells/well in IMDM medium containing 10% human AB serum. After 5 days, the cells were collected and plated with a new batch of Melan-A-pulsed irradiated CD8⁻ cells. After an additional 5 days the cells were collected and tested.

ELISpot Assays

Briefly, 96-well membrane bottomed plates (MAHA S4510; Millipore, Bedford, MA) were coated with 15 $\mu\text{g/mL}$ of antihuman IFN- γ antibody (Mabtech, Cincinnati, OH) in PBS. The plates were washed and CD8⁺ T cells, either freshly thawed at 5×10^4 cells/well or after in vitro expansion at 5×10^3 cells/well, were plated in triplicate in IMDM medium with 10% human AB serum. T2 cells (transporter associated with antigen processing-deficient cell line, American Type Culture Collection no. CRI 1992) were pulsed for 1 hour at 37°C with 50 $\mu\text{mol/L}$ peptide (either derived from HIV [ILKEPVHGV], Epstein-Barr virus [EBV; GLCTLVAML], or Melan-A [AAGIGILTV]), washed, and plated at a 5-to-1 ratio to the T cells. A replicate of CD8⁺ T cells was stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL) + ionomycin (0.5 $\mu\text{g/mL}$) as a positive control. After 24 hours, the cells were removed by washing with PBS + 0.05% Tween (wash buffer), and biotinylated antihuman IFN- γ antibody was added in PBS + 0.5% fetal calf serum. The plates were incubated for 2 to 4 hours at room temperature, washed, and streptavidin-alkaline phosphatase was added for 1 hour at room temperature. The plates were then washed, BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium) was added, and the plates were finally washed with water and allowed to air dry. Plates were scanned with an ELISpot reader (CTL Technologies, Cleveland, OH) and the number of spots per well was enumerated after the background was set on the basis of wells that had been incubated with medium alone; spot separation was adjusted using Immunospot software (CTL Technologies). For each sample, the number of T cells producing IFN- γ in response to EBV or Melan-A peptides was determined by subtracting the number of spots seen in response to HIV peptide. The mean and SD were determined for each triplicate sample. After immunization, the time point at which peak frequencies among the first three cycles were observed was used for data analysis.

Statistical Analysis

Comparisons between pre- and post-ELISpot frequencies were performed using a paired *t* test, and comparisons of augmented ELISpot frequencies between responders and nonresponders were made using an unpaired two-sided *t* test. Correlations between various dichotomous variables and clinical outcome were made using Fisher's exact test (two-sided). Survival data were determined using the Kaplan-Meier method, with differences among subgroups assessed by the log-rank test. All analyses were performed using SPSS software (version 8.0; SPSS Inc, Chicago, IL).

Table 2. Adverse Events

Adverse Event	Grade 1	Grade 2	Grade 3
Fatigue	16	0	0
Anorexia	6	0	0
Fever	7	0	0
Rash	3	0	0
Headache	3	0	0
Nausea	2	0	0
Injection site reaction	5	0	0
Neutropenia	1	2	0
Thrombocytopenia	2	0	0
Hepatic	5	2	0
Creatinine	1	0	0

NOTE. Adverse events were determined using the National Cancer Institute common toxicity criteria scale version 2.0.

RESULTS

Immunization Treatment and Toxicities

Each 3-week cycle consisted of immunization on day 1 and sc rhIL-12 administration on days 1, 3, and 5, as described in Methods. Three cycles constituted one course of therapy and patients were evaluated for response after each course. Patients were observed as inpatients in our General Clinical Research Center for the first 24 hours of each cycle.

Adverse reactions are listed in Table 2. All but one patient completed at least three cycles of therapy. There were no grade 3 to 4 toxicities; two patients had grade 2 neutropenia and two patients had grade 2 ALT or AST elevations, which were reversible. The most common adverse reactions were fatigue and fever.

Clinical Outcome

Clinical response outcomes are listed in Table 3. Two patients had a CR, for an overall response rate of 10%. In addition, four patients (20%) had a mixed response, one patient (5%) had an MR, four patients (20%) had SD, and the remaining nine patients (45%) had PD. The sites of tumor response were diverse. The two patients who experienced a CR both had numerous metastases of 2 cm or less and a normal LDH. One patient was female, had multiple cutaneous lesions, and no prior therapy; the other patient was male, had multiple lung lesions, and had experienced prior treatment failure from chemoimmunotherapy. Neither patient experienced a recurrence with a mean follow-up time of 28 months at the time of data analysis. Of the five other patients who showed a decrease in size of at least one tumor mass, three had responses in skin, one had a response in bone, and one had a response in an adrenal lesion. Three of the four patients with SD had visceral metastases.

Table 3. Clinical Outcome

Best Response	No. of Patients	%
Complete response	2	10
Partial response	0	0
Minor response	1	5
Mixed response	4	20
Stable disease	4	20
Progressive disease	9	45

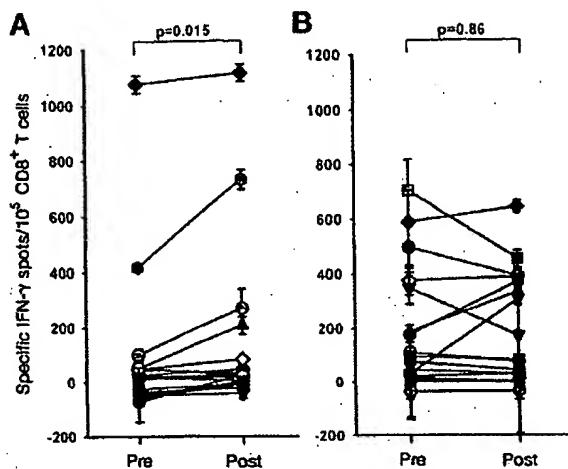


Fig 1. Interferon gamma ELISpot frequencies by CD8⁺ T cells against Melan-A and (A) Epstein-Barr virus (EBV) (B) pre- and postimmunization. Control values with HIV peptide were subtracted out. Post- and pretreatment values were compared using a paired *t* test.

Peptide-Specific T-Cell Responses by ELISpot

A carefully controlled IFN- γ ELISpot assay was used to monitor the immune response to immunization. Cryopreserved CD8⁺ T cells were thawed in batch fashion and stimulated in triplicate directly ex vivo with T2 cells loaded with peptides derived from either HIV, EBV, or Melan-A. The HIV values were subtracted from those obtained with either Melan-A or EBV as an internal control at each time point. Seventeen of the enrolled patients had adequate cryopreserved material with which to perform immunologic assessments.

As shown in Fig 1, some patients displayed a high frequency of Melan-A-specific CD8⁺ T cells before vaccination, with as high as 1% of CD8⁺ cells responding to this peptide. These T cells were functional because they produced IFN- γ . The majority of patients showed an increase in the frequency of Melan-A-specific cells after immunization ($P = .015$). In contrast, the frequencies of specific CD8⁺ T cells responding to the EBV peptide did not vary significantly overall ($P = .86$). Although the changes in T-cell frequency were modest, these results demonstrate an antigen-specific response after immunization with Melan-A peptide-pulsed PBMC + rhIL-12.

The changes in Melan-A-specific ELISpot frequencies were compared among patients who had a mixed response or better and those who had no clinical response. As shown in Fig 2, the mean increase in Melan-A-specific T cells for the clinical responders was 112 ± 45 and for nonresponders was 26 ± 16 , indicating that a greater absolute increase in Melan-A-specific T cells was associated with tumor regression ($P = .046$).

Survival and Associations Between Immunologic Parameters and Clinical Outcome

The overall median survival was 12.25 months and is shown in Fig 3A. Seven patients remained alive at the time of data analysis, with all patients followed beyond 12 months. Because the presence of elevated levels of serum LDH is a known

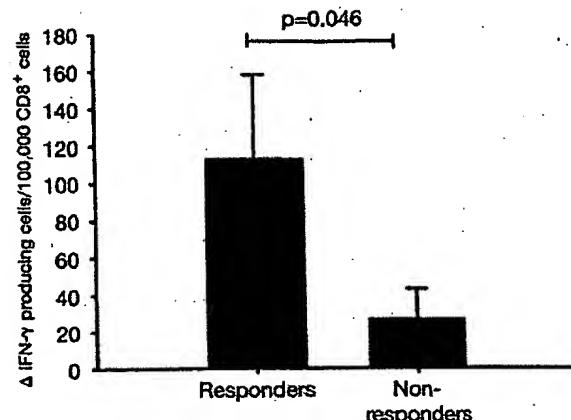


Fig 2. Comparison of increased Melan-A ELISpot frequencies after immunization between clinical responders and nonresponders. The absolute difference between Melan-A-specific ELISpot frequencies post- and pretreatment was compared between responders and nonresponders using a two-sided, unpaired *t* test.

negative prognostic factor,²³ survival was also compared in response to this vaccine on the basis of LDH level (Fig 3B). The median survival for patients with an elevated LDH level was 9.25 months, whereas the median had not yet been reached for those with a normal LDH ($P = .005$). In addition, the median survival for patients who experienced a significant increase in Melan-A-specific T cells was not yet reached, compared with 8.5 months for patients without a significant increase in Melan-A-specific cells (Fig 3C; $P = .120$).

Additional immunologic parameters that had been measured were also analyzed for associations with either clinical response or survival and are summarized in Table 4. Neither a positive recall DTH to standard antigens nor a relatively high number of EBV- or Melan-A-specific CD8⁺ T cells before immunization correlated with either outcome. The median pretreatment Melan-A-specific T cell frequency was 23 in clinical nonresponders and 26 in responders. To increase the sensitivity of the assay to detect Melan-A-specific T cells, an in vitro expansion was performed on the preimmunization samples and analyzed by ELISpot as described in Methods. Ten patients showed high Melan-A-specific T cell frequencies after in vitro expansion. However, this also failed to correlate with clinical outcome. Finally, although a normal LDH level was associated with survival, it did not correlate with clinical response and also did not correlate with immune response. Collectively, these results reinforce the specificity of the result showing a significant association between an increased number of Melan-A-specific T cells and clinical outcome.

Expression of Melan-A in Resected Tumors After Immunization

It was conceivable that some patients developed PD despite immunization because of outgrowth of Melan-A-negative tumor cells. Posttreatment tumor samples were obtained from progressing tumors from three patients and analyzed by RT-PCR. Although the new metastasis that developed in patient 1 was negative for

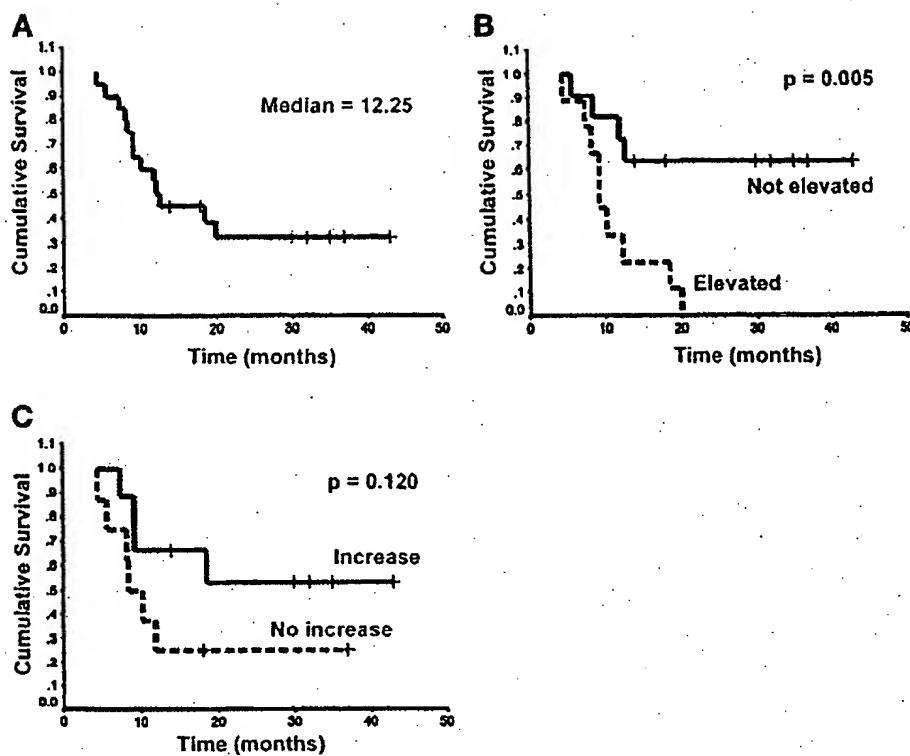


Fig 3. Overall survival for all patients (A), on the basis of serum lactate dehydrogenase greater than 200 U/L (B), and on the basis of increased Melan-A-specific interferon gamma-producing CD8⁺ T cells (C) was determined using the Kaplan-Meier method. Differences between groups were compared using the log-rank test.

Melan-A expression, those samples from patients 4 and 6 retained detectable expression of Melan-A mRNA (Fig 1). These results indicate that, although outgrowth of antigen-negative tumors can occur, other mechanisms of resistance to immune destruction likely explain the lack of clinical response in other patients.

DISCUSSION

In this study we used Melan-A peptide-pulsed autologous PBMC + rhIL-12 as a vaccine to treat HLA-A2-positive patients with advanced melanoma. We observed a significant increase in Melan-A-specific IFN- γ -producing CD8⁺ T cells after immunization, and found a statistical association between clinical response and the magnitude of the specific T-cell increase. Although it is difficult to compare across individual, small phase II studies, these results are similar to those that have been reported using antigen-loaded dendritic cells, but with a strategy that may be more straightforward to execute.

Preparation of the peptide-loaded PBMCs typically took 5 hours from phlebotomy to injection, and quality control of the cell product was facilitated by the lack of an extended *in vitro* culture period and absence of exposure to culture medium or serum proteins that is required for dendritic cell preparations. Conversely, dendritic cell vaccines have been prepared in batches and cryopreserved in individual doses in some studies, which obviates the need to prepare a fresh vaccine at each time point. Cryopreservation of vaccines has not yet been examined with our current approach. A comparative trial between PBMC/rhIL-12 and dendritic cell-based vaccination may, therefore, be of interest as the technologies continue to develop. Our results

support the notion developed in preclinical models that IL-12 can contribute to effective antitumor immunity, and are consistent with the results of a recent adjuvant vaccine study using rhIL-12 in melanoma.²⁴

We used a direct ex vivo ELISpot assay to assess antigen-specific T-cell responses in this study. Control experiments testing EBV reactivity from normal donors revealed that ELISpot analysis could be performed accurately on cryopreserved CD8⁺ T cell samples immediately after thawing (H. Harlin and T. Gajewski, unpublished data). We found that background reactivity against the control HIV peptide varied among patients and to some extent among time points for an individual patient. The magnitude of increase in apparent Melan-A-reactive T cells would have been greater in some patients had the values obtained with the HIV control peptide not been subtracted. We believe that this experimental detail is critical because it normalizes the samples for background differences and provides an internal control for minor variation between individual vials of cryopreserved T cells. We also compared the Melan-A frequencies to those against an EBV control peptide, to determine whether the treatment was altering ELISpot results. We performed our analyses on purified CD8⁺ T cells to control for variable numbers between patients and across time points. It is possible that we excluded subpopulations of CD8⁺ T cells, CD4⁺ T cells, and natural killer T cells that could have produced IFN- γ in response to Melan-A. Nonetheless, our results revealed a measurable and significant increase in Melan-A-specific T cells posttreatment. Our currently employed ELISpot assay is distinct from the assay used in our phase I trial of peptide-pulsed

Table 4. Statistical Correlates With Response or Survival

Parameter	Correlation With Response (P)	Correlation With Survival (P)
Positive DTH recall	.642	.130
Strong EBV pre-Rx*	.131	.491
Increased EBV post versus pret†	.290	.644
Strong Melan-A pre-Rx‡	.644	.481
Increased Melan-A post versus pret	.046	.120
Strong in vitro expansion of Melan-A§	.304	.565
LDH levels < 200	.99	.005

NOTE. Associations with response were determined using Fisher's exact test (two sided), except the differences between pre- and posttreatment, which were determined using an unpaired *t* test. Associations with survival were determined using the Kaplan-Meier method and log-rank test. Significant values are indicated in boldface.

Abbreviations: DTH, delayed-type hypersensitivity; EBV, Epstein-Barr virus; Rx, immunization; LDH, lactate dehydrogenase; HIV, human immunodeficiency virus; IL-2, interleukin-2.

*At least 90 spots per 10^5 CD8 $^+$ T cells after subtraction of background against a control HIV peptide.

†Changes between post- and prevaccination samples were calculated as the difference between the absolute number of specific spots and compared using an unpaired *t* test between clinical responders and nonresponders.

‡At least 40 spots per 10^5 CD8 $^+$ T cells after subtraction of background against a control HIV peptide.

§At least 90 spots per 10^5 CD8 $^+$ T cells after subtraction of background against a control HIV peptide, after a 10-day in vitro expansion with Melan-A peptide-pulsed autologous CD8 $^+$ cells and IL-2.

PBMC + rhIL-12 and in other trials^{21,25} in which in vitro expansion had been performed before assessment of IFN- γ production. Analysis of T-cell responses with minimal in vitro manipulation should most accurately reflect the status of those cells *in vivo*.

High frequencies of Melan-A-specific, IFN- γ -producing CD8 $^+$ T cells were observed in some patients at study entry when they clearly had progressively growing melanoma. This observation indicates that the absolute frequency of functional T cells against a tumor antigen does not correlate with the behavior of the tumor. We also found no statistical association between this high frequency and clinical outcome; in fact, the two patients who experienced a CR had undetectable Melan-A-specific T cells before therapy. Although high frequencies of T cells reacting with a Melan-A tetramer have been detected in some normal donors,²⁶ those cells had a naive surface phenotype and did not produce high levels of IFN- γ . What did correlate with clinical response in our current study is a meaningful increase in Melan-A-specific T cells posttreatment. These increases were modest (a net gain of 112 spots per 10^5 CD8 $^+$ T cells on average), indicating either that a subtle alteration in the steady-state between the immune response and a growing tumor in favor of increased T-cell frequencies is sufficient to translate into tumor regression, or that another immune function that we are not measuring is contributing to the final event of tumor shrinkage. Tumor regressions without detectable increases in

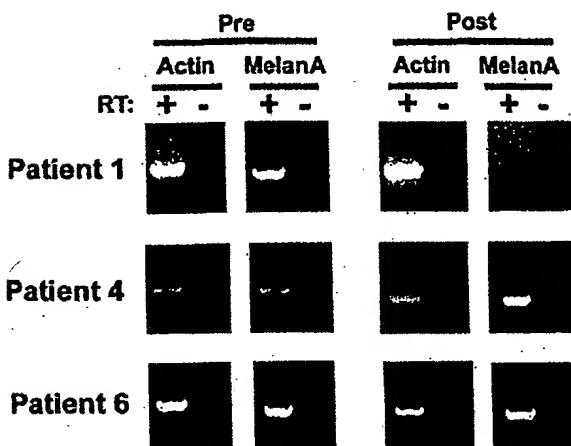


Fig 4. Melan-A expression in tumors that persisted after immunization. Three patients underwent surgical resection of lesions (after discontinuing the study), which were analyzed for Melan-A expression by qualitative reverse transcriptase polymerase chain reaction. Controls were analyzed without reverse transcriptase or with beta-actin primers.

T-cell frequencies using standard assays have been observed in other studies.²⁷

The median overall survival in our study was 12.25 months from treatment initiation, which is greater than the expected 6 to 9 months for this patient population. Although it was a relatively small study and subject to selection bias, most patients were pretreated and had visceral disease, one half of the patients had elevated serum LDH levels, and four patients had treated brain metastases. As has been seen in melanoma patients treated with standard therapies, we found that an elevated serum LDH level was a negative prognostic factor for survival. Whether this is reflective of tumor burden or the metabolic state of the tumor cells that have adapted to an anaerobic environment is unclear.

Some patients developed increases in Melan-A-specific T cells and developed progressive tumor growth despite retained expression of the antigen on posttreatment biopsies. This observation is similar to that seen in murine studies²⁸ and indicates mechanisms of tumor resistance downstream from initial T-cell priming, presumably within the tumor microenvironment. Potential explanations include poor T-cell trafficking to tumor sites, presence of negative regulatory cells, T-cell anergy or death, expression of inhibitory molecules by tumor cells, or downregulation of class I major histocompatibility complex or antigen-processing molecules.^{29,30} Future studies should investigate definable mechanisms of tumor escape that allow tumor cells to resist elimination by antigen-specific T cells *in vivo*.

ACKNOWLEDGMENT

We thank Genetics Institute/Wyeth for rhIL-12, and T. Garrison, M. Sherman, S. Swiger, and M. Posner for important contributions.

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Vaccination with Mage-3A1 Peptide-pulsed Mature, Monocyte-derived Dendritic Cells Expands Specific Cytotoxic T Cells and Induces Regression of Some Metastases in Advanced Stage IV Melanoma

By Beatrice Thurner,* Ina Haendle,* Claudia Röder,* Detlef Dieckmann,* Petra Keikavoussi,† Helmut Jonuleit,§ Armin Bender,* Christian Maczek,* Doris Schreiner,* Peter von den Driesch,* Eva B. Bröcker,‡ Ralph M. Steinman,|| Alexander Enk,§ Eckhart Kämpgen,‡ and Gerold Schuler*

*From the *Department of Dermatology, University of Erlangen-Nuremberg, D-91052 Erlangen, Germany; the †Department of Dermatology, University of Würzburg, D-97080 Würzburg, Germany; the §Department of Dermatology, University of Mainz, D-55131 Mainz, Germany; and the ||Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021*

Summary

Dendritic cells (DCs) are considered to be promising adjuvants for inducing immunity to cancer. We used mature, monocyte-derived DCs to elicit resistance to malignant melanoma. The DCs were pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin. 11 far advanced stage IV melanoma patients, who were progressive despite standard chemotherapy, received five DC vaccinations at 14-d intervals. The first three vaccinations were administered into the skin, 3×10^6 DCs each subcutaneously and intradermally, followed by two intravenous injections of 6×10^6 and 12×10^6 DCs, respectively. Only minor (less than or equal to grade II) side effects were observed. Immunity to the recall antigen was boosted. Significant expansions of Mage-3A1-specific CD8⁺ cytotoxic T lymphocyte (CTL) precursors were induced in 8/11 patients. Curiously, these immune responses often declined after the intravenous vaccinations. Regressions of individual metastases (skin, lymph node, lung, and liver) were evident in 6/11 patients. Resolution of skin metastases in two of the patients was accompanied by erythema and CD8⁺ T cell infiltration, whereas nonregressing lesions lacked CD8⁺ T cells as well as Mage-3 mRNA expression. This study proves the principle that DC "vaccines" can frequently expand tumor-specific CTLs and elicit regressions even in advanced cancer and, in addition, provides evidence for an active CD8⁺ CTL-tumor cell interaction *in situ* as well as escape by lack of tumor antigen expression.

Key words: dendritic cells • vaccination • active immunotherapy • melanoma • cytotoxic T lymphocytes

It is now established that the immune system has cells, particularly CD8⁺ CTLs, that can recognize tumor antigens and kill tumors (1, 2). Nevertheless, a major problem is that these T cells are either not induced or only weakly induced, i.e., the T cells are not evident in the systemic circulation. One possibility is that there is inadequate tumor antigen presentation by dendritic cells (DCs),¹ "nature's adjuvant" for eliciting T cell immunity (3). Another is that

tumor-reactive T cells are tolerized by the tumors (1, 4). Melanoma provides a compelling setting in which to pursue a current goal of cancer immunotherapy, the generation of stronger tumor-specific T cell immunity, particularly with CTLs (4). The majority of tumor antigens identified so far are expressed by melanomas (2). Limited antimelanoma CTL responses have been detected (5), and infusions of IL-2 expanded killer cells can lead to rejection of melanoma (6).

Conventional adjuvants promote antibody rather than CTL responses. Therefore, several novel strategies are being explored to induce tumor-specific T cell immunity. DC vaccination is one of these (3). Immature DCs capture

¹Abbreviations used in this paper: CNS, central nervous system; DCs, dendritic cells; DTH, delayed-type hypersensitivity; MCM, monocyte-conditioned medium; RT, reverse transcriptase; TT, tetanus toxoid.

antigens but lack full T cell-stimulatory activity (7). In the presence of appropriate stimuli, such as inflammatory cytokines, the DCs mature. DCs upregulate T cell adhesion and costimulatory molecules as well as select chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells. The use of DCs as adjuvants is supported by many animal experiments with primarily mature DCs (3, 8). These studies have shown that the injection of tumor antigen-loaded DCs reliably induces tumor-specific CTL responses, tumor resistance, and in some cases, regression of metastases (3, 8). In the few pilot trials reported so far for humans, immature DCs have been employed (9–11). Scattered tumor responses are reported, but evidence for the induction of tumor-specific CTLs by DC vaccination has not been shown.

We have developed a technique to generate large numbers of homogenous populations of mature and stable DCs from monocytes in the absence of nonhuman proteins (12, 13). We are now exploring the use of these DCs as vaccine adjuvants in humans. Here we provide the proof of the principle by demonstrating that three intracutaneous injections of Mage-3A1 peptide-pulsed mature DCs reliably enhance Mage-3A1-specific CD8⁺ and recall CD4⁺ T cell immunity in heavily pretreated, progressive stage IV melanoma patients with large tumor loads. Expansions of Mage-3A1-specific CTL responses have not been previously detected after Mage-3A1 peptide vaccination in less advanced melanoma patients (14), underscoring the potent adjuvant properties of DCs. As regressions of metastases also occurred upon DC-mediated immunization and were accompanied by CD8⁺ T cell infiltration, we propose that the induced Mage-3A1-specific CTLs are active *in vivo*.

Materials and Methods

Patient Eligibility Criteria

Patients were eligible if they suffered from stage IV (i.e., distant metastases) cutaneous malignant melanoma (1988 American Joint Committee on Cancer/Union Internationale Contre Cancer pTNM staging system) that was not curable by resection and was progressive despite chemo(immuno)therapy. Further inclusion criteria were an expected survival ≥ 4 mo, Karnofsky index $\geq 60\%$, age ≥ 18 yr, measurable disease, HLA-A1 positivity, expression of Mage-3 gene shown by reverse transcriptase (RT)-PCR in at least one excised metastasis, and no systemic chemo-, radio-, or immunotherapy within 4 wk (6 wk in the case of nitrosurea drugs) preceding the first DC vaccination. A positive skin test to recall antigens was *not* required. Important exclusion criteria were active central nervous system (CNS) metastasis, any significant psychiatric abnormality, severely impaired organ function (hematological, renal, liver), active autoimmune disease (except vitiligo), previous splenectomy or radiation therapy to the spleen, organ allografts, evidence for another active malignant neoplasm, pregnancy, lactation, or participation (or intent to participate) in any other clinical trial. Concomitant treatment (chemo- or immunotherapy, corticosteroids, investigational drugs, paramedical substances) was prohibited. Palliative radiation or surgical therapy of selected metastases and certain medications (acetaminophen/paracetamol, nonsteroidal anti-inflammatory drugs, opiates) to control symptoms were allowed.

Clinical Protocol and Study Design

The study was performed at the Departments of Dermatology in Erlangen, Würzburg, and Mainz, Germany according to standards of Good Clinical Practice for Trials on Medicinal Products in the European Community. The protocol was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research (New York, NY) and performed under supervision of its Office of Clinical Trials Management as study LUD #97-001. The protocol was also approved by the ethics committees of the involved study centers.

The study design is shown in Table II. All patients gave written informed consent before undergoing a screening evaluation to determine their eligibility. Extensive clinical and laboratory assessments were conducted at visits 1, 5, and 8 (Table II) and consisted of a complete physical examination, staging procedures, and standard laboratory values as well as special ones (pregnancy test, free testosterone in males, autoantibody profile, and antibodies to HIV-1/2, human T cell lymphotropic virus type I, hepatitis B virus, and hepatitis C virus). Patients were hospitalized and examined the day before each vaccination and were monitored for 48 h after the DC injections. Adverse events and changes in laboratory values were graded on a scale derived from the Common Toxicity Criteria of the National Cancer Institute, National Institutes of Health, Bethesda, MD.

Production of the DC Vaccine

During prestudy screening, we tested a small amount of fresh blood to verify that appropriate numbers of mature DCs could be generated from the patient's monocytes (12). Sufficient DC numbers could be successfully generated in all patients, but in some patients the test generation revealed that TNF- α had to be added to assure full maturation. To avoid repetitive blood drawings, we performed a single leukapheresis during visit 2 to generate DCs as described (13). In short, PBMCs from the leukapheresis ($\geq 10^{10}$ nucleated cells) were isolated on Lymphoprep™ (Nycomed Pharma) and divided into three fractions. The first fraction of 10^9 PBMCs was cultured on bacteriological petri dishes (Cat. #1005; Falcon Labware) coated with human Ig (100 μ g/ml; Sandoglobin™; Sandoz GmbH) in complete RPMI 1640 medium (BioWhittaker) supplemented with 20 μ g/ml gentamicin (Refobacin 10; Merck), 2 mM glutamine (BioWhittaker), and 1% heat-inactivated human plasma for 24 h to generate monocyte-conditioned medium (MCM) for later use as the DC maturation stimulus. The second fraction of 3×10^8 PBMCs was used for the generation of DCs for vaccination 1 and delayed-type hypersensitivity (DTH) test I. Adherent monocytes were cultured in 1,000 U/ml GM-CSF (10×10^7 U/mg; Leukomax™; Novartis) and 800 U/ml IL-4 (purity >98%; 4.1×10^7 U/mg in a bioassay using proliferation of human IL-4R $^+$ CTLL; CellGenix; expressed in *Escherichia coli* and produced under good laboratory practice conditions but verified for good-manufacturing practice [GMP] safety and purity criteria by us) for 6 d, and then MCM was added to mature the DCs. MCM was supplemented in patients 04, 06, 09, 11, and 12 with 10 ng/ml GMP-rhu TNF- α (purity >99%; 5×10^7 U/mg in a bioassay using murine L-M cells; a gift of Dr. G.R. Adolf, Boehringer Ingelheim Austria, Vienna, Austria) to assure full maturation of DCs. Mature DCs were harvested on day 7. The third fraction of PBMCs was frozen in aliquots and stored in the gas phase of liquid nitrogen to generate DCs for later vaccinations and DTH tests.

DCs for vaccinations were pulsed with the Mage-3A1 peptide (15), (EVDPIGHLY, synthesized at GMP quality by Clinalfa) as tumor antigen, and as a recall antigen and positive control, tetanus toxoid (TT) or tuberculin (if at visit 1 the DTH to TT in the

Multitest Merieux was >10 mm; both purchased from the Bacterial Vaccines Department of the Statens Serum Institute, Copenhagen, Denmark). The recall antigen was added at 10 µg/ml for the last 24 h, and the Mage-3A1 peptide was added at 10 µM directly to the cultures for the last 8 h (if immunity to recall antigens was strongly boosted, the dose of recall antigen was reduced to 1.0 or 0.1 µg/ml or was omitted for the intravenous DC injections to avoid a cytokine release syndrome). On day 7, mature DCs were harvested, resuspended in complete medium, washed, and pulsed once more with Mage-3A1 peptide (now at 30 µM) for 60 min at 37°C. DCs were finally washed and resuspended in PBS (GMP quality PBS; BioWhittaker) for injection. DCs to be used for Mage-3A1 DTH tests were pulsed with Mage-3A1 (but no recall antigen); DCs that served as negative control in the DTH tests were not pulsed at all. An aliquot of the DCs to be used for vaccinations was analyzed as described (13) to assure that functionally active and mature DCs were generated. The features of the DCs are described in Results. Release criteria were typical morphology (>95% nonadherent veiled cells) and phenotype (>95% HLA-DR⁺⁺⁺CD86⁺⁺⁺CD40⁺CD25⁺CD14⁻ and >65% homogenously CD83⁺⁺).

Immunization Schedule

A total of five vaccinations (three into the skin followed by two intravenously) with antigen-pulsed DCs were given at 14-d intervals (Table II). This design was chosen to explore the toxicity and efficacy of various routes in this trial. For vaccinations 1–3, 3×10^6 DCs were given subcutaneously at two sites (1.5×10^6 DCs in 500 µl PBS per site) and 3×10^6 intradermally at 10 sites (3×10^5 DCs in 100 µl PBS per site). The injection sites were the ventromedial regions of the upper arms and the thighs close to the regional lymph nodes and were rotated clockwise. Limbs where draining lymph nodes had been removed and/or irradiated were excluded. For intravenous vaccinations 4 and 5, a total of 6 and 12×10^6 antigen-pulsed DCs (resuspended in 25 or 50 ml PBS plus 1% autologous plasma) was administered over 5 and 10 min, respectively. Premedication with an antipyretic (500 mg acetaminophen/paracetamol p.o.) and an antihistamine (2.68 mg clemastinhydrogenfumarat i.v.) was given 30 min before intravenous DC vaccination.

Evaluation of Immune Status

Recall Antigen-specific Proliferation and Cytokine Production. PBMCs were cultured in triplicate at two dose levels (3×10^4 and 1×10^5 PBMCs/well) plus or minus TT or tuberculin (at 0.1, 1, and 10 µg/ml) and pulsed on day 5 with [³H]thymidine for 12 h. In all cases, the highest cpm's were obtained with the highest doses of PBMCs and antigen and are shown in Fig. 2. IL-4 and IFN-γ levels were measured in culture media by ELISA (Endogen, Inc.). In a separate plate, staphylococcal enterotoxin (SEA; Serva) was added at 0.5, 1, and 5 ng/ml, and proliferation was assessed after 3 d to provide a positive control for helper T cell viability and responsiveness.

Enzyme-linked Immunospot Assay for IFN-γ Release from Single Antigen-specific T Cells. To quantitate antigen-specific, IFN-γ-releasing, Mage-3A1-specific effector T cells, an enzyme-linked immunospot (ELISPOT) assay was used as described (16). PBMCs (10^5 and 5×10^5 /well) or in some cases CD8⁺ or CD4⁺ T cells (isolated by MACS™ according to the manufacturer, Miltenyi Biotech) were added in triplicate to nitrocellulose-bottomed 96-well plates (MAHA S4510; Millipore Corp.) precoated with the primary anti-IFN-γ mAb (1-D1K; Mabtech) in 50 µl ELISPOT

medium (RPMI 1640 and 5% heat-inactivated human serum) per well. For the detection of Mage-3A1-reactive T cells, the APCs were irradiated T2A1 cells (provided by P. van der Bruggen, Ludwig Institute of Cancer Research, Brussels, Belgium) pulsed with MHC class I-restricted peptides (Mage-3A1 peptide and the HIV-1 p17-derived negative control peptide GSEELRSLY) added at 7.5×10^4 /well (final volume 100 µl/well). After incubation for 20 h, wells were washed six times, incubated with biotinylated second mAb to IFN-γ (7-B6-1; Mabtech) for 2 h, washed, and stained with Vectastain Elite kit (Vector Labs.). For detection of TT-reactive T cells, TT was added at 10 µg/ml directly to the PBMCs (1 or 5×10^3 PBMCs/flat-bottomed 96-well plate). Assays were performed on fresh PBMCs. Spots were evaluated and counted using a special computer-assisted video imaging analysis system (Carl Zeiss Vision) as described (16).

Semiquantitative Assessment of CTL Precursors. The multiple microculture method developed by Romero et al. (17) was used to obtain a semiquantitative assessment of CTLp (precursors) specific for Mage-3A1 peptide. Aliquots of frozen PMBCs were thawed and assayed together. CD8⁺ T cells were isolated with magnetic microbeads (MACS™ separation columns; Miltenyi Biotech) and seeded at 10^4 /well in 96-well round-bottomed plates in RPMI 1640 with 10% heat-inactivated human serum. The CD8⁺ PBMCs were pulsed with peptide Mage-3A1 or the influenza PB1 control peptide VSDGGPNLY (10 µg/ml; 30 min at room temperature), irradiated (30 Gy from a cesium source), and added as an APC population at 10^5 /well together with IL-2 (10 IU/ml final) and IL-7 (10 ng/ml final) in a total volume of 200 µl/well. On day 7, 100 µl fresh medium was substituted, and peptide Mage-3A1 or PB1 (1 µg/ml final) and IL-2 (10 U/ml) was added. On day 12, each microwell was divided into three equal samples to test cytolytic activity in a standard 4-h ⁵¹Cr-release assay on peptide-pulsed (10 µg/ml for 1 h at 37°C) T2A1 cells, nonpulsed T2A1 cells, and K562 target cells, respectively. All of the assays were performed with an 80-fold excess of nonlabeled K562 to block NK activity. Microwells were scored positive if lysis of T2A1 targets with peptide minus lysis without peptide was $\geq 12\%$ and this specific lysis was greater than or equal to twice the lysis of T2A1 targets without peptide plus six as described (18). We aimed at testing 30 microwells of 10^4 CD8⁺ T cells. Therefore, 1/30 positive wells equals at least one CTLp in 3×10^5 (i.e., 30 wells at 10^4 CTLp per well) CD8⁺ T cells (corresponding to $\sim 3 \times 10^6$ PBMCs).

DTH. DTH to Mage-3A1 peptide was assessed by intradermal injection at two sites of each 3×10^5 Mage-3A1 peptide-loaded DC in 0.1 ml PBS. Negative controls were nonpulsed autologous DCs in 0.1 ml PBS and 0.1 ml PBS. DTH to seven common recall antigens (Multitest Merieux) including TT and tuberculin was performed on visits 1, 5, and 8 (Table II).

Assessment and Analysis of Tumor Tissue

For recruitment into the study, Mage-3 gene expression in at least one metastatic deposit had to be demonstrated by RT-PCR as described (14). Accessible superficial skin metastases were removed whenever possible after the vaccinations and subjected to Mage-3 RT-PCR as well as routine histology and immunohistochemistry (to characterize cellular infiltrates).

Statistical Analysis

For analysis of the immune response, pre- and postimmunization values were compared by paired t test after logarithmic transformation of the data. Significance was set at $P < 0.05$.

Results

Patient Characteristics

All 13 patients were HLA-A1+, had proven Mage-3 mRNA expression in at least one excised metastasis, and suffered from advanced stage IV melanoma, i.e., distant metastases that were progressive despite chemotherapy and, in some cases, chemoimmunotherapy (Table I). We offered DCs to all patients who fulfilled the inclusion and exclusion criteria, i.e., we did not select for subsets of patients. Two patients (numbers 01 and 03) succumbed to melanoma after two and three vaccinations, respectively. 11 patients received all five planned DC vaccinations in 14-d intervals (Table II) and were thus fully evaluable.

Quality of the Vaccine

All vaccine preparations were highly enriched in mature DCs. More than 95% of the cells were large and veiled in

appearance, expressed a characteristic phenotype by flow cytometry (HLA-DR+++CD86+++CD40+CD25+CD14-), and acted as strong stimulators of an MLR at DC/T cell ratios of $\leq 1:300$ (13). Most (mean 80%) expressed the CD83 mature DC marker (19). These features were stable upon removal of cytokines and culture for one to two more days (13). The DCs were pulsed with Mage-3A1 peptide as a tumor antigen and TT or tuberculin as a recall antigen. The latter were internal controls for immunization and possibly provided help for CTL responses (20).

Toxicity

No major (above grade II) toxicity or severe side effects were observed in any patient, including the two patients who were not fully evaluable. We noticed, however, local reactions (erythema, induration, pruritus) at the intracuta-

Table I. Patients' Characteristics, Status before DC Vaccination, and Response to DC Vaccination

Patient code	Sex/Age	Onset stage IV	Previous therapy	Metastases at study entry*								Clinical Response 14 days after the 5 th vaccination	Survival		
				regional		distant									
				skin	LN	Skin	LN	Lung	Liver	Other					
Patients with objective tumor regression															
04	M48	1/98	PCI									CNS reg**	complete regression of all but 1 lung metastasis, overall progression	10+>9	
06	F61	10/97	CI										complete regression* of 1 lung + 4 s.c. ^a metastases, overall progression	6+>16	
07	F48	6/97	C										complete regression* of 1 lung ^b + 2 s.c. ^a metastases, overall progression	13+12†	
08	M67	11/97	PC										complete regression* of lung + liver + 4 s.c. ^a metastases, overall progression**	8+3†	
09	F43	5/98	C										Partial regression of 1 lung metastasis, overall progression	4+>11	
12	M54	9/96	CI										partial regression of axillary LN metastases, overall progression	26+>9	
Patients without objective tumor regression															
02	F73	5/96	PCI	150/40									continuous progression	18+5†	
05	F49	10/97	CI		150								continuous progression	5+>17	
10	M62	8/98	C	150/70		150							continuous progression	1+6†	
11	F72	7/98	C		150/20	150							continuous progression	4+9†	
13	M34	12/97	CI					150					continuous progression	12+5†	

Treatment centers: three patients (04, 08, and 12) were treated in Wuerzburg, two in Mainz (patients 10 and 13), and the others in Erlangen.

Pretreatment therapy: PCI, polychemoimmuno. Preceding excisions and radiotherapies are not listed.

Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in millimeters). m, multiple (>3 metastases).

Survival: (since onset of stage IV and as of 5 August 1999) is listed as months since onset stage IV until study entry + number of months since study entry. ^aPatient deceased.

*CNS metastases were regressing at study entry after gamma knife treatment.

^bDeveloped (in part) after study entry.

^cDetermined by autopsy.

^dSudden death from bleeding into CNS metastasis on visit 8.

^eThe regressions of lung metastases in patients 06 and 07 were documented at a staging 3 mo after visit 8. mediast., mediastinum; pancr., pancreas.

Table II. Study Design

Activities	Screen	Leuka pheresis	Vacc. #1 3 Mio s.c. 3 Mio i.d.	Vacc. #2 3 Mio s.c. 3 Mio i.d.	Vacc. #3 3 Mio s.c. 3 Mio i.d.	Vacc. #4 6 Mio i.v.	Vacc. #5 12 Mio i.v.	Final Evaluation
Clinical visit	1	2	3	4	5	6	7	8
Day	-28/-14	-9	+1	+14	+28	+42	+56	+70
Vaccination			X	X	X	X	X	
Multitest Meneux	X				X			X
DTH to Mage-3A1 peptide-loaded DC			X		X		X	
Recall antigen proliferation		X						X
CTLC analysis		X				X		X
ELISPOT		X	X	X	X	X	X	X
Mage-3A1								
ELISPOT recall antigen		x	x	x	x	x	x	x

X, prespecified in the protocol as obligatory; x, optional.

neous vaccination sites that increased with the number of vaccinations. In 9/11 patients, strong DTH reactions (induration >10 mm in diameter) were noted to DCs carrying a recall antigen (Fig. 1). Elevation of body temperature (grade I and II fever) was observed in most (9/11) patients and was also related to pulsing DCs with recall antigen. The most striking example was patient 02, who progressively developed fever (up to 40°C) upon successive vaccinations but did not show a rise in body temperature when TT was omitted for the final (fifth) vaccination. We observed slight lymph node enlargement, clinically in 63% and by sonography in 83% of patients, after the intracutaneous DC injections. Interestingly, these were delayed, being inapparent during the 2 d of monitoring after vaccinations but detected when patients were investigated again the day before the next vaccination (Table II).

Immunological Responses

Boosting of Recall Antigen-specific Immunity. PBMCs that had been frozen before vaccination and 14 d after vaccination 5 were thawed and assayed together, as specified in the protocol (Table II). In most patients, a significant boost of antigen-specific immunity developed to TT (and tuberculin in patient 10) ($P < 0.004$; Fig. 2). Supernatants from the proliferative assays contained large amounts of IFN- γ (mean 1,679 pg/ml, range 846–4,325) but little IL-4 (IFN- γ /IL-4, 317:1). In five patients, we studied the kinetics of the immune response to TT by IFN- γ ELISPOT analysis. We found an increase after the intracutaneous vaccinations ($P < 0.02$) but a peculiar decrease after the intravenous vaccinations ($P < 0.008$; Fig. 3). Thus, comparing recall immunity before and after all five vaccinations (Fig. 2) as prespecified in the protocol (Table II) obviously underestimated the extent of boosting.

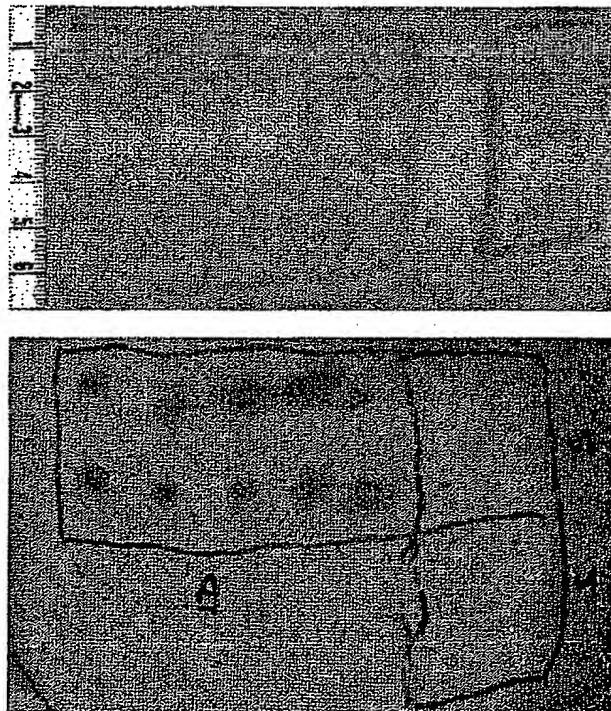


Figure 1. Local reactions to DCs carrying Mage-3A1 peptide and TT at the intradermal and subcutaneous vaccination sites in patient 09 (24 h after vaccination 2; top panel) and 02 (48 h after vaccination 3; bottom panel). Erythema at the 10 intradermal (left) and 2 subcutaneous (right) vaccination sites was followed by induration >10 mm in diameter (with secondary purpura in patient 02). These local reactions represent strong DTH reactions to DCs carrying TT, as such strong reactions did not occur in response to unpulsed DCs or DCs pulsed with Mage-3A1 peptide alone in DTH tests I–III (Table II; reactions not shown).

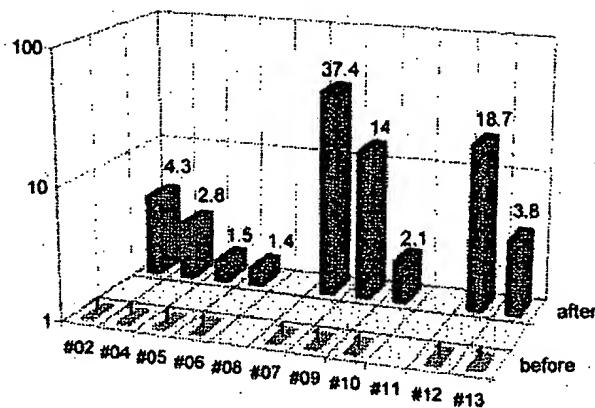
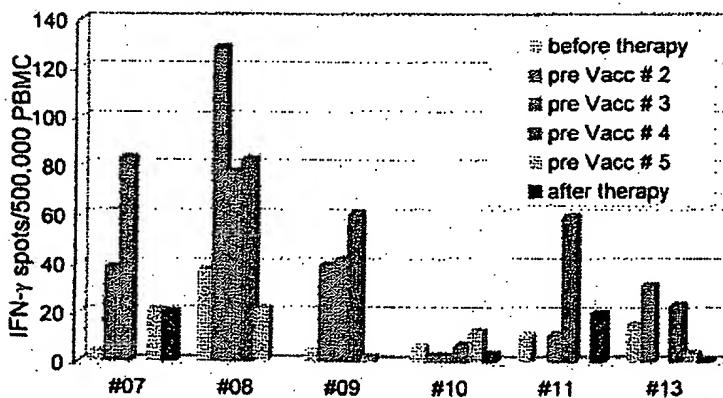


Figure 2. Recall antigen-specific immunity (tuberculin in patient 10; TT in all others) as assayed by antigen-specific proliferation. The cpm values determined after therapy (14 d after vaccination 5) are shown as multiples of pretherapy cpm values. Absolute cpm (cpm with recall antigen minus cpm without antigen) after therapy was 68,917 in patient 02, 85,225 in patient 04, 16,759 in patient 05, 7,913 in patient 06, 16,367 in patient 07, 107,923 in patient 09, 22,790 in patient 10, 4,507 in patient 12, and 1,831 in patient 13 (SEM for all measurements was <20%). Patients 08 and 11 could not be evaluated due to shortage of cells after therapy.

Expansion of Mage-3A1-specific CTLp. Aliquots of PBMCs, frozen before the first and after the third and fifth vaccinations, were thawed at the same time (Table II) and subjected to a semiquantitative recall assay for CTLp (reference 17; Fig. 4). Before vaccination, CTLp frequencies were low or undetectable. In 8/11 patients, we found a significant expansion of Mage-3A1-specific CTLp as indicated by the increase (mean, eightfold; $P < 0.008$) of positive microcultures in the multiple microculture procedure employed for the semiquantitative assessment of CTLp (17). Interestingly, in six patients, the CTLp frequencies were maximal after the three intracutaneous vaccinations ($P < 0.0013$) but then decreased after the two additional intravenous vaccinations in all but one of these patients ($P < 0.026$). Only in 1/11 patients did we observe an increase of CTLp to an irrelevant PB1 influenza peptide that served as a specificity control (not shown).



ELISPOT Analysis for IFN- γ -releasing, Mage-3A1-specific T Cells. We also tried to detect Mage-3A1-specific CTL effectors in uncultured fresh, nonfrozen PBMCs by performing ELISPOT analyses at 14-d intervals on all patients. A significant increase of Mage-3A1-reactive IFN- γ spot-forming cells was apparent only in patients 07 and 09 after the first and second vaccinations, respectively, but the frequency of Mage-3A1-specific effectors was very high (~5,000 and 10,500/10⁷ CD8⁺ T cells; not shown).

DTH Test to Mage-3A1 Peptide-loaded DCs. Tests of DTH to Mage-3A1 peptide-loaded DCs yielded erythema and/or induration (>5 mm diameter) in 7/11 patients (not shown). The results were, however, equivocal due to the frequently observed background to nonpulsed DCs (up to 10 mm in diameter) and the variability from test site to test site.

Clinical Responses

At the end of the trial, i.e., ~2 wk after the fifth vaccination (Table II), we observed temporary growth cessation of some individual metastases, but more intriguingly, in 6/11 patients, complete regression of individual metastases in skin, lymph nodes, lung, and liver (Table I and Fig. 5). Resolution of skin metastases was found in three patients (Table I, patients 06, 07, and 08) and in two of them (06 and 07). It was preceded by local pain, itching, and slight erythema. The six regressing skin lesions of patients 06 and 07 (Table I) were also excised and examined by immunohistology. Clusters of CD8⁺ T cells were seen around and in the tumor, the latter often necrotic, suggesting an immune attack (Fig. 6).

In patients 06 and 08, the metastases excised at study entry (four and two, respectively) proved to be Mage-3 mRNA⁺. However, all of the samples removed at the end (two and six, respectively) were Mage-3 mRNA⁻, suggesting immunoselection for antigen-negative tumor cells. Remarkably, significant infiltration of CD8⁺ T cells was not found in any of these lesions.

Discussion

In deciding on the source of DCs for this phase I trial, we selected mature, monocyte-derived DCs for the follow-

Figure 3. Kinetic analysis of immunity to recall antigens as assessed by TT-specific IFN- γ ELISPOT (SEM for all measurements was <20%). Blood was drawn (see Table II, Study Design) before the first DC vaccination and then every 14 d just before administration of the next DC vaccination (e.g., pre Vacc # 2 means immediately before vaccination 2, i.e., 14 d after vaccination 1), and finally after therapy. Time points at which vaccinations were not performed lack bars. Note the increase after the intracutaneous vaccinations and the decline upon the two vaccinations after intravenous ones. Patient 10, who received tuberculin-pulsed DCs, exhibited no significant change in the TT-specific IFN- γ ELISPOT as expected.

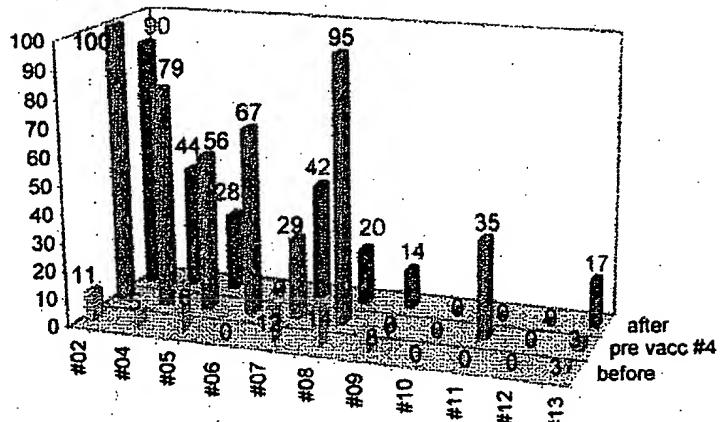


Figure 4. Mage-3A1 CTLp frequency analysis as assessed by semiquantitative recall assay. The y-axis and the numbers above the bars indicate the percentage of positive wells found before vaccination 1, before vaccination 4 (14 d after vaccination 3), and after therapy (usually 14 d after vaccination 5).

ing reasons. Monocyte-derived DCs currently represent the most homogenous and potent DC populations, with several defining criteria and quality controls (12, 13, 21). The method for generating production of these DCs is very reproducible and allows the cryopreservation of large numbers of cells at an identical stage of development (12, 13). Furthermore, these DCs can be produced in the absence of potentially hazardous FCS (12, 13, 21). FCS exposure also leads to large syngeneic T cell responses in culture, so their clinical use (11) might produce nonspecific immunostimulatory effects. Unlike other investigators (9–11), we chose to use mature rather than immature DCs for our first melanoma trial. The DCs that have been used with efficacy in animal experiments were primarily mature (3, 8). Mature DCs are much more potent in inducing CTL and Th1 responses *in vitro* (reference 22 and Jönuleit, H., A. Gieseke, A. Kandemir, L. Paragnik, J. Knop, and A.H. Enk, manuscript in preparation), and the DCs are also resistant to the immunosuppressive effects of IL-10 (23) that can be produced by tumors (24–26). Mature DCs also display an extended half-life of antigen-presenting MHC class I (26a) and class II molecules (27). Finally, mature DCs have a high migratory activity (21) and express CCR7 (28), a receptor for chemokines produced constitutively in

lymphoid tissues (28). Mature DCs, as used in this cancer therapy trial, have recently also been shown to rapidly generate broad T cell immunity in healthy subjects (28).

Mature DCs were loaded with only one melanoma peptide, Mage-3A1, to avoid uncertainties regarding loading of DCs with multiple peptides (11) of varying affinity and off rate. Successful loading was verified with a Mage-3A1-specific CTL clone and ELISPOT analysis (not shown). The Mage-3A1 peptide (15) was selected for several reasons. It is essentially tumor specific (2) and expressed in tumors other than melanoma (2), and the Mage-3A1 epitope is likely a rejection antigen (14). Moreover, the Mage-3A1 CTLp frequency is exceedingly low in noncancer patients (reference 18; 0.4–3 per 10^7 CD8 $^{+}$ T cells) as well as in cancer patients, even after peptide vaccination (14). Thus, any induction or boost of Mage-3A1 CD8 $^{+}$ T cell responses would indicate a significant superiority in the adjuvant capacities of DCs.

DTH assays with peptide-pulsed DCs were carried out as described by Nestle et al. (11) to detect Mage-3A1 immunity (not shown). However, we did not detect unequivocal DTH. This was due to the frequently observed background to nonpulsed DCs (possibly due to cytokine production by DCs) and the noteworthy variability from test site to test site. As Mage-3A1-specific T cells are CD8 $^{+}$

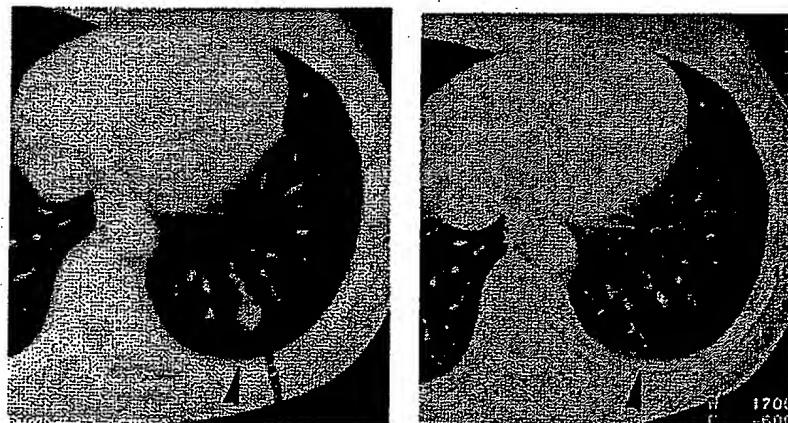


Figure 5. Regression (arrows) of a globular (13 mm in diameter) lung metastasis in patient 07 that was then no longer detectable in serial 6-mm-thick computed tomography scans.

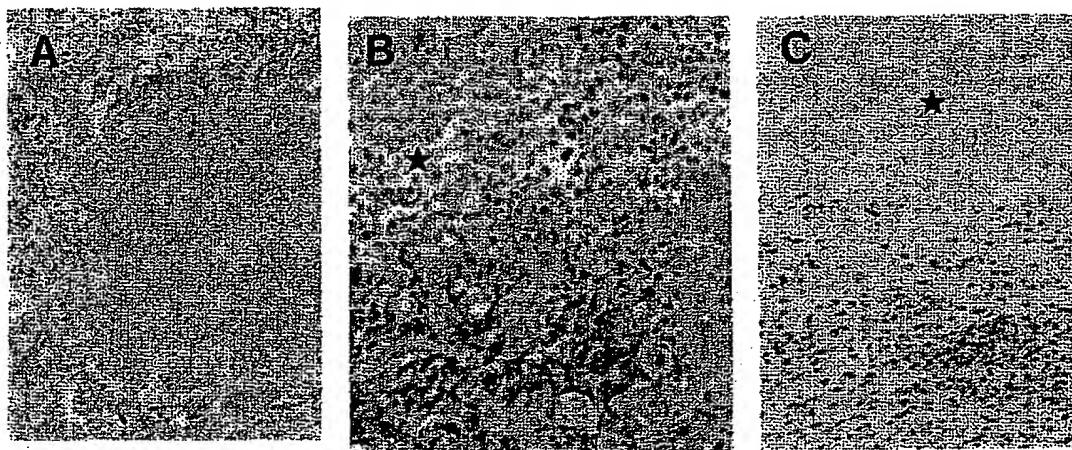


Figure 6. Regressing subcutaneous metastases in patient 06 display a CD8⁺ lymphocytic infiltrate (alkaline phosphatase/antialkaline phosphatase immunohistochemical staining with anti-CD8 mAb) that surrounds (A) and infiltrates (B) the tumor. Areas of damaged (B, *) and necrotic (C, **) melanoma cells are obvious in the vicinity of the CD8⁺ T cell infiltrate. The metastasis expressed Mage-3, as demonstrated by RT-PCR (data not shown). Magnifications: A, 100; B, 250; C, 160.

T cells and DTH assays typically detect primed CD4⁺ T cells, we suspect that DTH to MHC class I peptide-pulsed DCs may also for this reason prove not to be a sensitive or reliable way to monitor specific CD8⁺ T cell-mediated immunity.

In contrast, we found sizable expansions of Mage-3A1-specific CTL precursors in PBMCs from a majority (8/11) of patients ($P < 0.008$; Fig. 4). This is an important proof of the principle of DC-based immunization, and it is also significant from the point of view that tumors can induce tolerance or anergy. It is very promising that CTLp expansions can be induced in far advanced and heavily pretreated stage IV melanoma patients. However, active Mage-3A1-specific effectors were generally not observed in ELISPOT assays, except for in two patients with high frequencies ($>5,000/10^7$ CD8⁺ T cells). Perhaps active CD8⁺ effectors were rapidly sequestered in the numerous metastases, as suggested by the biopsy studies illustrated in Fig. 6. An alternative explanation is that looking for effectors in peripheral blood 14 d after a preceding vaccination might simply be too late.

Interestingly, in six patients, CTLp had increased to their highest levels after the three intracutaneous vaccinations ($P < 0.0013$) and then decreased ($P < 0.026$) with subsequent intravenous immunizations (Fig. 4). The decrease in CTLp might be due to emigration of activated Mage-3-reactive CTLs into tissues, tolerance induction, or clonal exhaustion via the intravenous route. We also observed decreased responses to recall antigens in the five patients that we studied before and after intravenous vaccination (Fig. 3). The effect of the intravenous route requires additional study, as it may be counterproductive. In contrast, our results clearly demonstrate that the intracutaneous route is effective, so that the less practical intranodal injection propagated by other investigators (11) does not seem essential. It will, however, be necessary to compare subcutaneous and intradermal routes to find out if one is superior.

We found regression of individual metastases in 6/11 patients when patients were staged 14 d after the fifth vaccination (Table I). This percentage of responses was unexpected in far advanced stage IV melanoma patients who were all progressive despite standard chemotherapy and even chemoimmunotherapy. In the study by Nestle et al. (11), chemotherapy was only given to 4/16 melanoma patients, and objective tumor responses were observed in 5/16. Therefore, we attribute the regressions to DC-mediated induction of Mage-3A1-specific CTLs. This interpretation is supported by the heavy infiltration with CD8⁺ T cells of regressing but not nonregressing (skin) metastases. The observation that all of the metastases in patients 06 and 08 that were excised at the end of the study were Mage-3 mRNA⁻ (whereas those removed at the onset were uniformly positive) suggests immune escape of and selection for Mage-3 antigen-negative tumors. Immune escape might also have been responsible for the lack of tumor response in those nonresponders that had mounted a Mage-3A1-specific CTL response.

After the end of the trial, surviving patients received further vaccinations with DCs and several tumor peptides (Mage-1, tyrosinase, and Mage-3) that were no longer part of the protocol. It is encouraging that 5/11 patients are still alive (Table I) 9–17 mo after study entry, as the expected median survival in patients progressive after chemo(imuno)therapy is only 4 mo (29, 30). One of the initial responders (patient 06) has recently experienced a complete response and has now been disease free for 2 mo. It is interesting that Marchand et al. (14) have also observed that regressions, once they have started, proceed slowly and may take months to complete.

In conclusion, the use of a defined DC vaccine combined with detailed immunomonitoring provides proof that vaccination with mature DCs expands tumor-specific T cells in advanced melanoma patients. In addition, we have found some evidence for the direct interaction between

CD8⁺ CTLs and tumor cells as well as for escape of antigen-negative metastases. We are convinced that DC-mediated immunization can be intensified further to reveal the presence of expanded populations of effector cells. Efficacy might be increased at the level of the DC, e.g., by optimizing

variables such as DC maturational state, route, dose, and schedule or by improving the short life span of DCs *in vivo* (31, 32); at the level of the T cell, e.g., by providing melanoma-specific CD4⁺ T cell help (33, 34) or IL-2 (35); and by treating patients earlier in their disease course.

We are grateful to all patients for their confidence and cooperation. We appreciate the support of J. Knop, Head of the Department of Dermatology in Mainz. We thank our colleagues H.B.-R. Balda, H. Hintner, F.S.M. Meurer, and C.R. Neumann for referring patients and T.L. Diepgen for statistical analysis. We are particularly grateful to T. Boon and P. van der Bruggen, who helped us in many ways, and to A. Knuth and T. Woelfel for help in establishing the semiquantitative CTL and ELISPOT assays, respectively. We are obliged to the Protocol Review Committee and Office of Clinical Trials Management of the Ludwig Institute for Cancer Research, in particular H.F. Oettgen and E. Hoffman, for their suggestions on improving the protocol and for supervising the trial.

This work was supported by a grant from the Deutsche Krebshilfe (project #70-2291). B. Thurner was supported by the Training and Mobility of Researchers (TMR) programme of the European Union (EUNIDI).

Address correspondence to Gerold Schuler, Dermatologische Klinik mit Poliklinik, Hartmannstr. 14, D-91052 Erlangen, Germany. Phone: 49-9131-85-0, ext. 33661; Fax: 49-9131-85-6175; E-mail: schuler@derma.med.uni-erlangen.de

Submitted: 1 June 1999 Revised: 21 September 1999 Accepted: 24 September 1999

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		Filing Date	NOVEMBER 20, 2001
		First Named Inventor	AVI J. ASHKENAZI
		Group/Art Unit	1647
		Examiner Name	HAMUD, FOZIA M.
Total Number of Pages in This Submission	129	Attorney Docket Number	39780-2730 P1C71

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